The Role of Suppressor of Cytokine Signaling 1 and 3 in Human Cytomegalovirus Replication

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der Universität Zürich

von

Olmo Sonzogni

von

Moleno, TI

Promotionskomitee:

Prof. Dr. Nicolas J. Müller
(Leitung der Dissertation)

Prof. Dr. Christoph Renner
(Vorsitz)

Prof. Dr. Amedeo Caflisch

Zürich, 2012

To my parents

Table of contents

1. Summary

Human cytomegalovirus (HCMV) is a large encapsulated virus belonging to the herpesvirus family. Following asymptomatic infection, life-long latency is established in the host and 50-80% of the general population is seropositive. This virus can cause life-threatening infections in immunocompromised patients after transplantation, in neonates or in people infected with human immunodeficiency virus. Primary infection or reactivation of HCMV are major causes of morbidity and mortality.

Suppressor of cytokine signaling (SOCS) proteins are very potent negative regulators of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. JAK/STAT are key players for numerous immunological relevant pathways including the interferon (IFN) pathway, one of the most powerful cellular antiviral defense mechanism. A number of viruses have been described to exploit SOCS signaling to attenuate the immune response of the infected cell and facilitate replication. Two herpesviruses, herpes simplex virus 1 and Epstein Barr virus, upregulate SOCS1 and in particular SOCS3 to their advantage. Based on this findings, we hypothesized that HCMV may take advantage of SOCS1 or/and SOCS3 as an evasion mechanism. The data presented in this thesis describe the process of investigation that led to the final results demonstrating that efficient replication of HCMV in primary endothelial cells is depended on SOCS3 protein. First experiments were carried out in porcine endothelial cells. The goal was to evaluate possible modulation of SOCS proteins by HCMV infection in a cross-species model of xenotransplantation. After observing a modulation of SOCS1 mRNA in porcine primary endothelial cells we focused on the human setting, using our established model of HCMV infection in primary endothelial cells.

Time course analysis of HCMV infected human endothelial cells revealed an increase of SOCS3 protein in infected cells. Silencing of SOCS3 resulted in a partially inhibited production of viral antigens. Consistently, the number of infectious particles produced in SOCS3 silenced (siSOCS3) cultures was significantly lower as compared to SOCS1 silenced (siSOCS1) or control transfected cultures (siCNTR). Impairment of the viral replication process due to SOCS3 silencing occurred after entry since the number of infected cells was identical in all three siSOCS1, siSOCS3 or siCNTR conditions. STAT1 phosphorylation, a key transcription factor for type I

and II IFN, was increased in siSOCS3 and siSOCS1 infected cells when compared to siCNTR-treated cells. In contrast, phosphorylation of STAT2, key factor of type I IFN, was only increased in siSOCS3 infected-cultures. Analysis of the ability of the virus to control IFNβ-inducible gene expression showed that the HCMV-infected fraction of siSOCS3 culture had a higher percentage of cells expressing IFNβ-induced major histocompatibility complex class I molecules as compared with siSOCS1 or siCNTR cultures.

In conclusion this study demonstrates for the first time that SOCS3 plays a critical role for efficient HCMV replication in primary endothelial cells and provides evidence for an involvement of SOCS3 in HCMV-mediated control of the type I IFN pathway.

2. Zusammenfassung

Humane Zytomegaloviren (HZMV) gehören zur Familie der Herpesviren. Die Infektion ist in der Regel asymptomatisch, und führt zur Latenz, d.h. lebenslangen Persistenz im Wirt. 50%-80% der allgemeinen Bevölkerung sind seropositiv für HZMV. Dieses Virus ist besonders relevant für Menschen mit einer angeborenen oder erworbenen Immunschwäche, wie transplantierte Patienten, Neugeborene und Personen, die mit dem humanen Immunodefizienz Virus infiziert sind. Sowohl die Erstinfektion als auch die Reaktivierung von HZMV resultiert in einer erheblichen Morbidität und Mortalität.

Suppressor of Cytokine Signaling (SOCS)-Proteine sind sehr potente negative Regulatoren des Janus-Kinase/Signal-Wandler und Aktivatoren der Transkription (JAK/STAT) Kaskaden. JAK/STAT spielen eine wichtige Rolle in vielen immunologisch relevanten Signalwegen, unter anderem der Interferon (IFN)-Kaskade, die eine der potentesten zellulären antiviralen Abwehrmechanismen darstellt. Einige Viren sind beschrieben worden, die SOCS-Proteine ausnützen, um die Immunantwort der infizierten Zellen zu dämpfen und damit ihrer Replikation zu erleichtern. Bei zwei Herpesviren, Herpes simplex Virus 1 und Epstein Barr Virus konnte gezeigt werden, dass diese SOCS1 und SOCS3 zu ihrem Vorteil regulieren. Auf dieser Grundlage haben wir die Hypothese formuliert, dass HZMV SOCS1 oder/und SOCS3 für die eigene Replikation moduliert. Die hier präsentierten Resultate zeigen, dass eine effiziente Replikation von HZMV in primären Endothelzellen massgebend vom SOCS3 Protein abhängt.

Die ersten Experimente wurden in porzinen Endothelzellen durchgeführt. Diese Erkenntnisse sollten helfen, dass Risiko einer Spezies-übergreifenden Infektion besser abzuschätzen. Dieses Szenario ist bis jetzt nur in wenigen klinischen Studien Realität, bei denen Diabetes Patienten porzine Inselzellen verabreicht werden.

Die Modulierbarkeit der SOCS Gene in porzinen Endothelzellen motivierte uns, die Situation in einem menschlichen Modell genauer zu untersuchen. Dabei benützten wir das in unserem Labor entwickelte Modell der HZMV Infektion menschlicher Endothelzellen.

HZMV infizierte menschliche Endothelzellen zeigten eine Zunahme von SOCS3 Protein. Das gezielte Ausschalten des SOCS3 Genes (silencing, siSOCS3) führte zu einer teilweise gehemmten Produktion von viralen Antigenen. Als Folge war die

Anzahl der infektiösen Partikel in siSOCS3 Kulturen deutlich niedriger verglichen mit siSOCS1 oder kontroll-transfizierten Kulturen (siCNTR).
Die deutlich eingeschränkte Replikation in siSOCS3 Kulturen trat erst nach Eintritt des Virus in die Zelle auf, da die Zahl der infizierten Zellen in allen drei Versuchsanordnungen (siSOCS1, siSOCS3 oder siCNTR) identisch war. STAT1 Phosphorylierung, ein wichtiger Transkriptionsfaktor für Typ I und II IFN war in siSOCS3 und siSOCS1 infizierten Zellen erhöht im Vergleich mit den siCNTR-behandelten Zellen. Im Gegensatz dazu wurde die Phosphorylierung von STAT2, Schlüsselfaktor des Typ I IFN, nur in siSOCS3 infizierten Kulturen beobachtet.
Die Analyse der durch das Virus kontrollierten IFNß-induzierbaren Genexpression zeigte, dass die HCMV-infizierten siSOCS3 Kulturen einen höheren Prozentsatz von Zellen zeigte, die IFNß-induzierte Haupthistokompatibilitätskomplex Klasse I Moleküle exprimieren als in siSOCS1 oder siCNTR Kulturen. Diese Studie zeigt zum ersten Mal, dass SOCS3 für eine effiziente Replikation vom HZMV in primären Endothelzellen eine entscheidende Rolle spielt. HZMV hemmt dabei durch SOCS3 die Typ I IFN Antwort und begünstigt dadurch die eigene Replikation.

3. Introduction

3.1 Human cytomegalovirus

3.1.1 Epidemiology and clinical relevance

Human cytomegalovirus (HCMV) primary infection is mainly asymptomatic. Life-long latency is established after infection, resulting in a seropositivity of 50-80% of the general population. This is of relevance for immunocompromised hosts like neonates, patients infected with human immunodeficiency virus or recipients after solid and stem cell transplantation. HCMV primary infection and reactivation is a major cause of mortality [1] in this vulnerable population. Despite a worldwide effort no vaccination has yet reached the clinical routine. In the 1970s first treatment options with antiviral drugs showed a decrease in mortality after HCMV infection [2]. Currently marketed anti-CMV drugs are ganciclovir, its oral prodrug valganciclovir, foscarnet, cidofovir and fomivirsen. Except for fomivirsen, which is an antisense RNA targeting expression of immediate early gene 72 and is only applied intraocularly, the other drugs target the viral DNA polymerase. Prolonged treatment with these drugs has led to emerging resistant viral strains [3,4]. Moreover, toxicity limits duration and applicability. Alternative antiviral compounds with new mechanisms of action, such as artesunate, leflunomid, letermovir and maribavir, are now being investigated in clinical studies. An advantage of some of the new substances is a favorable toxicity profile, which might lead to new prophylactic and treatment strategies [5]. HCMV remains a highly investigated pathogen and every step elucidating its biology may potentially lead to the development of new treatment options and improve outcome after infection.

3.1.2 Structure and replication cycle

HCMV is a big enveloped virus, which belongs to the β-herpesvirus family, and is known as herpes virus type 5. The virion has an icosahedral structure and contains a 230Kb double stranded DNA genome [1]. The envelope is made out of 6 viral glycoproteins (gB, gH, gL, gM, gN and gO) that are responsible for virus docking and cellular entry. Infection of HCMV occurs by an interaction between the cellular

heparan sulfate proteoglycans and the viral proteins gB and gM complex (Figure 3.1). Epidermal growth factor (EGF) receptor is believed to be involved in the entry process, however the data are controversial [6]. Platelet-derived growth factor-α receptor has been shown to be important for HCMV infection of fibroblast, epithelial and endothelial cells [7]. After entry into the target cells the viral genome rapidly reaches the nucleus.

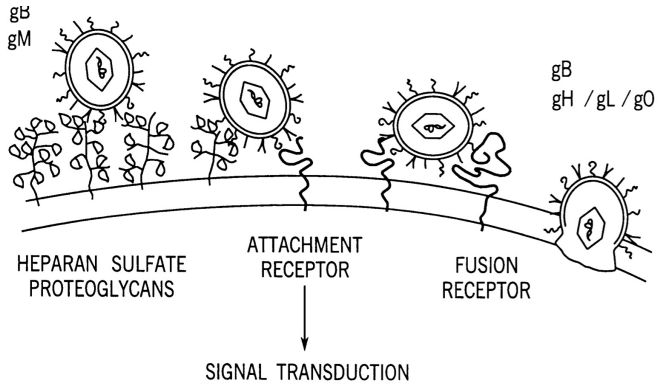

Figure 3.1: Model of HCMV entry into the host cell

The diagram shows HCMV entry into human cells. Initial attachment occurs by binding of viral envelope gB to heparan sulfate proteoglycans. This low-affinity attachment is followed by high affinity attachment of gB to an unidentified receptor. Finally, in a step which depends on a viral glycoprotein complex of gH, gL, and gO, the lipid bilayers fuse and the virion is released into the cytoplasm (Adapted from [8]).

HCMV genome encodes for more than 200 proteins. Expression of these proteins is divided in three overlapping phases: immediate early (IE), early (E) and late (L) (Figure 3.2). IE antigens expression occurs very rapidly after entry, within the first 2-4 hours. These proteins are responsible for activation of later viral gene transcription and regulate expression of numerous cellular genes affecting the immune response of the host cell and clearance mechanisms [9,10]. E antigens expression starts within the first 24 hours after infection and depends on IE expression. These proteins are responsible for viral genome replication and are involved in immune evasion as well [10]. Expression of L proteins takes place at the final stage of the infection, beginning 48 hours post infection (p.i.) and is depending on viral genome replication. These proteins are mainly structural proteins required for virus assembly and budding [10-12]. Finally mature viral particles are released starting 72 hours p.i. by an exocytic-like pathway [12].

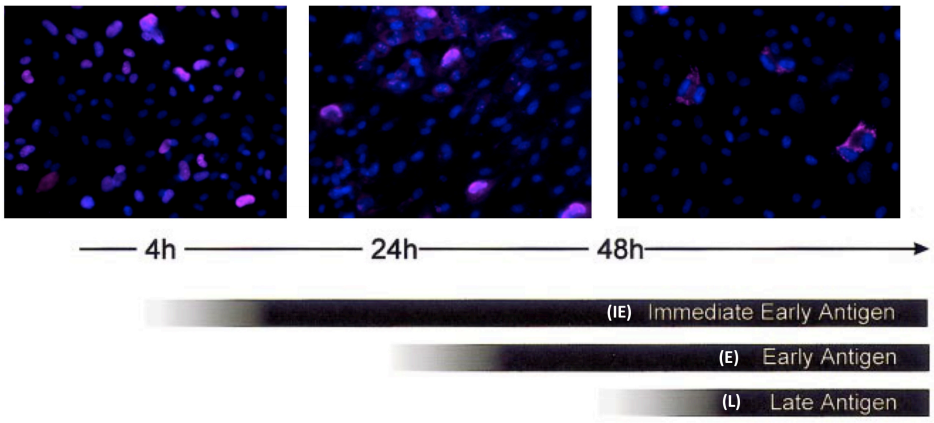

Figure 3.2: Steps of viral antigens production during HCMV replication
Detection of viral antigen in infected cells starts 2-4 hours p.i. with IE antigens, followed about 24 hours p.i. by E antigens and 48-72 hours p.i. by L antigens. [13], immunofluorescence images from our laboratory show IE (left), E (middle) and L (right) staining.

3.1.3 Role in allo- and xenotransplantation

Patients undergoing solid organ transplantation have to be immunosuppressed to reduce the possibility of rejection of the graft. In this context HCMV infection poses a danger as reactivation or primary infection by transmission of HCMV from a seropositive donor to a seronegative recipient may result in disease. HCMV infection has been correlated with rejection for all type of solid organ transplants [14-21]. It can affect the donor in a direct or indirect manner. The direct effect (called CMV disease) is characterized by a high-rate viral replication leading to pneumonitis, gastrointestinal disease, hepatitis, retinitis and encephalitis [22]. The indirect effects of HCMV are characterized by a low rate of replication over a long period of time. This has been associated with a higher risk of graft rejection and opportunistic infections and may lead to the development of diabetes and arteriosclerosis [22,23].
In view of the currently performed clinical trials using porcine islets for diabetic patients as well as large animal studies performed with the goal of implanting pig organs into human recipients (xenotransplantation), investigations in the pig to baboon model has revealed both direct and indirect effect of pig and baboon CMV reactivation, resulting in death of some of the baboons [24]. Recently HCMV infection of porcine endothelial cells (pEC) was demonstrated [25] and further characterized [26,27]. These studies not only demonstrated that HCMV can enter and actively

replicate in pEC leading to production of infectious particles but did show an impact on the phenotype of infected cells. In view of the potential pig to human transplantation of solid organs, it is important to investigate HCMV infection of pEC to evaluate the impact and possible effects.

3.1.4 The role of the endothelium

HCMV can productively infect a number of different cells including but not limited to epithelial, endothelial, smooth muscle and mesenchymal cells, as well as monocyte-derived macrophages, fibroblasts, granulocytes and hepatocytes [28]. In the context of transplantation the endothelium plays a crucial role. Being at the interface between blood and tissue the endothelium is the first barrier and contact surface between donor graft and recipient blood. It has been shown to be a natural site of infection for HCMV *in vivo* following primary infection [29-33] and it is believed to act as a viral reservoir [34] involved in viral spread and persistence. Virus-mediated changes in the phenotype of infected endothelial cells (EC) may contribute to the dissemination of the virus into organs and has been associated with viral pathogenicity. [35].
Given the combined importance, the EC were chosen for our model.

3.1.5 Multifaceted escape mechanisms form host immune defense

Following HCMV infection both innate and adaptive immune responses are elicited. The innate response starts within minutes after infection of the cell and induces the expression of inflammatory genes, most of them belonging to the interferon (IFN) family [6,10]. HCMV has developed a number of strategies to elude innate responses which vary depending on the virus strain and the cell type studied [9].
HCMV has been shown to interfere with type I and II IFN (Figure 3.3) activation at different levels. Again, the observed alterations are highly dependent on the model investigated [9]. Characterization of fibroblast infection with the HCMV laboratory strain Towne, which is commonly used in many models, has revealed inhibition of type II IFN at different levels after infection, starting with the inhibition of class II transactivator expression, about 6h p.i. [36], followed by the inhibition of signal transducer and activator of transcription 1 (STAT1) phosphorylation by src homology region 2 domain-containing phosphatase, about 16h p.i. [37], and finally disruption of janus kinase 1 (JAK1) 72h p.i. [38]. Downregulation of IFNγ-induced major histocompatibility complex class II (MHC-II) expression in HCMV infected cells has

been shown [36,38-41]. JAK1 disruption by HCMV has an influence on type I IFN activation since it is a shared component of both the type I and II IFN pathway (Figure 3.3).

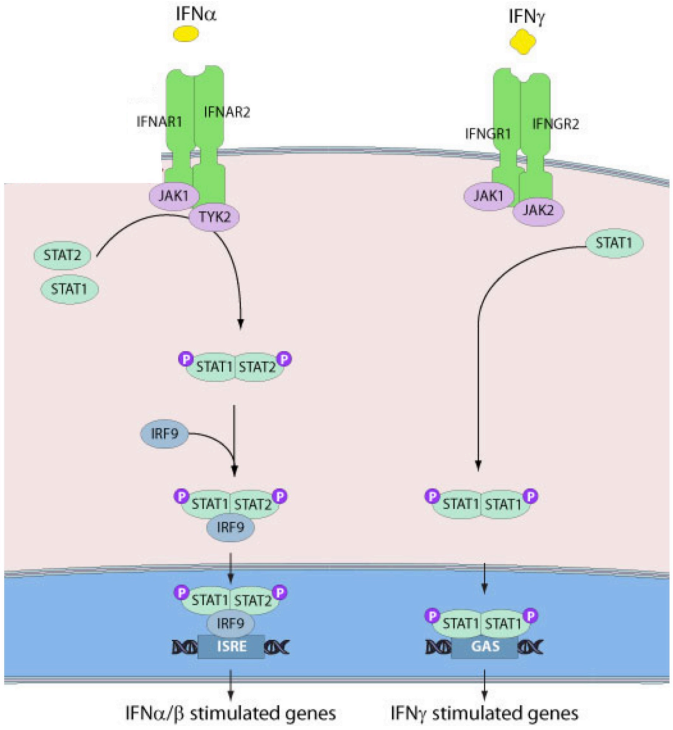

Figure 3.3: Type I and II IFN pathways

A simplified summary of type I and II IFN pathway activation is shown. IFN type I (most important members are IFNα and IFNβ) and type II (only member is IFNγ) pathways start after cytokine docking to the receptor. After engaging JAK1 and TYK2 for type I IFN and JAK1 and JAK2 for type II IFN, the cascade leads to STAT1/STAT2 or STAT1/STAT1 phosphodimers generation, respectively. Once the dimers translocate to the nucleus (with the help of cofactors like IFN regulatory factor-9 (IRF9) they finally activate the transcription of the target genes (Adapted from the Swiss Institute of Bioinformatic http://viralzone.expasy.org/all_by_protein/784.html)

In addition to JAK1, p48, another key factor of the type I IFN pathway, has been found to be destroyed in HCMV infected cells [42]. HCMV can directly affect the type I IFN pathway, inhibiting IFN type I stimulated MHC class I, IFN regulatory-1, myxovirus resistance A (Mx) and 2,5-oligoadenylate synthetase genes expression in fibroblast and human umbilical vein endothelial cells (HUVEC) [9,42]. These results are in conflict with others showing an upregulation of these genes in HCMV infected fibroblasts [43-49].

It was recently shown that in HCMV infected fibroblast a decrease in STAT2 phosphorylation is followed by a reduction in the total amount of this protein. This phenomenon was found to be strain depended and not observed for Towne virus [50]. An active involvement of E viral proteins in the control of STAT2 has been suggested [50]. More recently, in fibroblast, a physical interaction of STAT2 and the viral protein IE72 has been observed which was connected to the ability of the virus to control the STAT2 activation [51].

The innate response rapidly limits viral replication and induces the adaptive arm. Antibodies against HCMV persist lifelong after infection [1]. Nevertheless, HCMV is capable of establishing life-long latency [52]. Escape from the adaptive response depends on the ability of the virus to induce expression of genes that interfere with the mechanisms of cellular mediated clearance [1,53,54]. Expression of unique short proteins (e.g. US2, US3, US6 and US11) lead to human leukocyte antigen (HLA) class I and II degradation, inhibition of transcription or internalization and as consequence T cell-mediated recognition of infected cells is inhibited [55]. Natural killer (NK) cell response is inhibited by the viral protein gpUL40, which induces HLA-E molecule expression in infected cells. HLA-E binds to NK cells receptor inhibiting the cytotoxic effect of this cells thus protecting the infected cells from NK cell-mediated clearance [56,57]. The viral proteins gpUL16 and gpUL141 interfere with NK cells by binding the NK activator receptor natural killer group 2 member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1), leading to inhibition of the NK killing process [58-61]. HCMV can efficiently interfere with the apoptotic process promoting the survival of infected cells [54,62]. Inhibition of apoptosis has been shown to be one of the main barriers for cross species infection (HCMV to pEC). HCMV is very inefficient in controlling this pathway probably because of differences, between human and pig, in the structure of cellular proteins involved in this process [26,63].

3.2 Suppressor of cytokine signaling proteins

3.2.1 The origins

In 1995 a protein called CIS (cytokine-inducible SH2) was identified by differential expression in the presence of interleukin 3 (IL-3) and erythropoietin (EPO) [64]. Two years later three independent groups discovered a second member of the same family, called suppressor of cytokine signaling 1 (SOCS1) protein via three different approaches: based on the homology with STAT3 central domain [65], based on a yeast-two-hybrid screen reveling interaction with JAK2 tyrosine kinase JH1 domain [66] and as inhibitor of IL-6 induced macrophage cell line differentiation [67]. These three first publications revealed the common role for SOCS1 as an inhibitor of JAK/STAT related signals [65-67].

Eight members of the SOCS protein family have been identified so far: CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7 [67-69]. Their main role is to modulate the response of cells to cytokines by attenuating the activation of target pathways, usually JAK/STAT family [70]. They are particularly important to restore the homeostasis after activation by cytokines (e.g. IFNγ, IL-6) [71] acting as inhibitors of the JAK/STAT pathway in a negative feedback loop manner leading to the attenuation of the transduction signals [65,71,72].

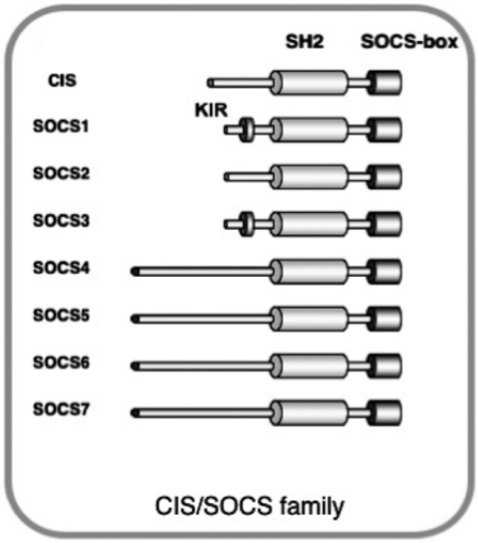

Figure 3.4: Suppressor of cytokine signaling proteins structure

SOCS proteins all share the same basic structure composed by an N-terminal domain which is variable in length and sequence, an SH2 domain and a C-terminal domain called SOCS box. SOCS1 and SOCS3 in addition show a kinase inhibitor region (KIR) (adapted from [73]).

3.2.2 Structure and function

SOCS proteins are characterized by their particular composition. They all share the same structure with an SH2 and a C-terminal SOCS box motive (Figure 3.4). Indeed, after the identification of CIS and SOCS1, the other member of this family were identified through database searches for proteins that contained a SH2 and SOCS box [68,69].

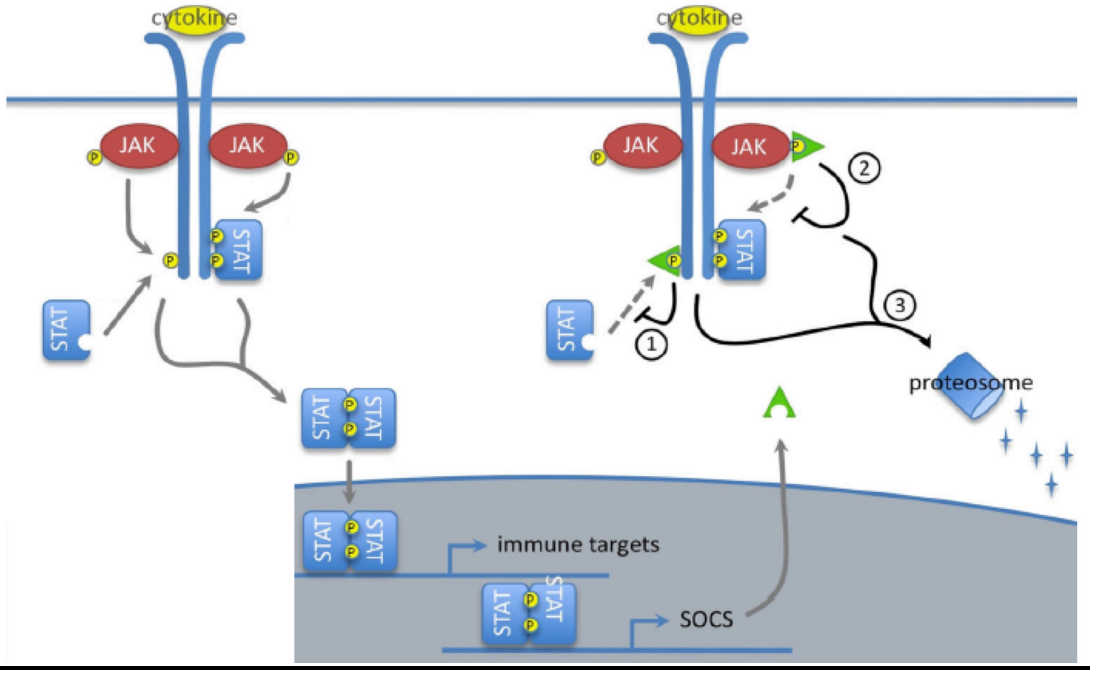

Figure 3.5: SOCS and JAK/STAT pathway

Once a cytokine binds to its receptor activation of the JAK/STAT pathways occurs. This leads to the transcription of target genes, including SOCS. SOCS protein act in a negative feedback manner attenuating the transduction of the pathway by (1) competing with STAT for the phosphotyrosine residue on the receptor, (2) inhibiting JAK activity (by KIR, only for SOCS1 and SOCS3) or binding the target proteins with the SH2 domains allowing polyubiquitination and consequent destruction in the proteasome (adapted from **[74]**).

The function is tightly related to the structure: the SOCS proteins attenuate the transduction and activation of JAK/STAT related pathways [75]. The SH2 domain binds to a phosphorylated tyrosine residue of the target protein, usually a kinase. The SOCS box is a binding site for the ubiquitin machinery [76]. Once a target protein is bound to the SH2 domain (e.g. JAK) the ubiquitin machinery allows polyubiquitination, targeting the SH2 recognized protein for proteasomal degradation (Figure 3.5). SOCS1 and SOCS3 have an additional particular sequence, called

kinase inhibitor region (KIR), which is located close to the N-terminal part of the SH2 domain. KIR can directly inhibit the kinase activity of target proteins. Therefore SOCS1 and SOCS3 do not only inhibit the transduction of the signal through SOCS box mediated polyubiquitination but can directly interact with the kinase activity of the target protein [75].

3.2.3 SOCS features

Out of the 8 proteins belonging to this family SOCS1 and SOCS3 are the most investigated members followed by CIS and SOCS2. SOCS4, SOCS5, SOCS6 and SOCS7 are in contrast not very well characterized so far and seem to be less involved in control of cytokines signaling as compared to SOCS1 and SOCS3. Investigations of SOCS family proteins have revealed that SOCS proteins with short N-terminal region (CIS, SOCS1, SOCS2 and SOCS3) are associated with disease states, whereas long terminal region does not seem to involved in this context [77-79]. Most research has focused on short N-terminal region SOCS, mainly SOCS1 and SOCS3. They play a key role in a number of biological processes and have been described to be relevant in a broad range of fields, like cancer biology [80-82] , immunology [83,84], inflammation [85,86] and virology [74]. A table (Table 3.1) summarizes the stimuli that induce the SOCS expression and the pathway affected. The following chapter highlights the main findings of the first 15 years after their discovery.

3.2.3.1 SOCS1

SOCS1 is mainly induced by type I and II IFN but not exclusively (Table 3.1). Studies in mice have shown that at birth SOCS1 deficient mice ($SOCS1^{-/-}$) were found to be healthy but smaller than wild-type mice. Thereafter these knock out mice show activation of peripheral T-cells, lymphopenia, macrophage infiltration in many tissues, fat degeneration and necrosis of the liver which results in death within three weeks [87,88]. These effects are thought to be due to a dysregulation of the immune system. In these mice STAT1 was found to be constitutively activated; in mice lacking type II IFN receptor and SOCS1, 3 weeks after birth, the lethal phenotype was not observed. These findings suggest that SOCS1 is a crucial negative regulator of type II IFN pathway [89-91]. However, despite an initial protection, type II IFN

receptor and SOCS1 mutated mice still died after 6 weeks of life, developing inflammation and polycystic kidneys [92].

Table 3.1: Factor involved in SOCS expression and regulation (Adapted from [93])
Abbreviations: IGF-1, insulin-like growth factor-1; LIF, leukemia inhibitory factor; M-CSF, macrophage-colony stimulating factor; G-CSF, granulocyte colony stimulation factor; CNTF, ciliary neuronotrophic factor; GH, growth hormone; PAMPs, pathogen-associated molecular patterns; TPO thrombopoietin; TSLP, thymic stromal lymphopoietin; TSH, thyroid stimulating hormone; GM-CSF granulocyte-macrophage colony-stimulating factor; other explained in the text and in the abbreviation list.

SOCS family	Inducer/inhibitor	Immunoregulatory cytokines	Colony stimulating factors	Hormones and growth factors
CIS	Induced by Inhibits	IL-2, IL-3, IL-6, IL-9, IFN-α, TNF-α IL-2, IL-3	EPO, TSLP EPO	GH, prolactin GH, prolactin
SOCS-1	Induced by Inhibits	IL-2, IL-4, IL-6, IL-7, IL-9, IL-13, IFN-α/β, IFN-γ, LIF, TNF-α IL-2, IL\L-4, IL-6, IL-7, IL-12, IL-15, IFN-α/β, IFN-γ, LIF, TNF-α	EPO, TPO, TSLP, G-CSF, GM-CSF, M-CSF EPO, TPO, TSLP	GH, prolactin, insulin, CNTF, cadiotropin, TSH GH, prolactin, insulin, leptin
SOCS-2	Induced by Inhibits	IL-6, IFN-α, IFN-γ, LIF IL-6		GH, prolactin, insulin, CNTF, cadiotropin GH, IGF-1
SOCS-3	Induced by Inhibits	IL-1, IL-2, IL-6, IL-9, IL-10, IL-13, IFN-α, IFN-γ, LIF IL-2, IL-4, IL-6, IL-9, IL-11, IFN-α/β, IFN-γ, LIF	EPO, GM-CSF EPO	GH, prolactin, insulin, leptin, CNTF GH, prolactin, insulin leptin
SOCS-4	Induced by Inhibits	Unknown		
SOCS-5	Induced by Inhibits	IL-6 IL-4, IL-6		
SOCS-6	Induced by Inhibits	Unknown		
SOCS-7	Induced by Inhibits	Unknown		

Interestingly SOCS1$^{-/-}$ mutant mice restricted to the hematopoietic compartment resulted in neonatal death. Constitutive activation of STAT1 and high expression of type II inducible genes were detected, suggesting that the hematopoietic stem cells plays a crucial role in the development of the lethal phenotype [94].
SOCS1 mRNA is transcribed by STAT1, STAT3 and STAT6 [65,95] and needs methylation of the CpG island within the promoter. The translation efficiency of SOCS1 mRNA is regulated by the 5' untranslated region which offer alternative starting points [96,97]. Emerging relevance of microRNA-155 (miR-155) as negative regulator of SOCS1 has been recently published [98-103]. Depending on the cell type mIR-155 inhibits the transcription or the translation of SOCS1 [102]. Expression

of miR-155 is induced by toll like receptor (TLR) signals (TLR2, 3, 4 and 9) and by stimulation (IL-1, tumor necrosis factor α (TNFα), phorbol 12-myristate 13-acetate (PMA), IFNβ and γ) indicating that this SOCS1 modulator is expressed in the context of inflammation [104-108].

It has been shown that the SOCS box domain of SOCS1 plays a role in the stability of the protein. A SOCS box deletion mutant of SOCS1 was protected from proteasomal degradation [109-111]. This phenomenon is probably due to the fact that the ubiquitin machinery is not able to bind the SOCS box deleted mutant of SOCS1 [70,112]. In line with this concept, once the SOCS1 SOCS box is phosphorylated by the Pim family serine/threonine kinase the binding of the ubiquitin machinery is blocked resulting in prolongation of the half-life of the protein [113].

3.2.3.2 SOCS3

Expression of SOCS3 is induced particularly but not exclusively by IL-6 and type I IFN (Table 3.1). In mice it has been shown that SOCS3 knock-out mice died in embryonic state due to placenta dysfunction [114,115]. Conditional knock-out of hematopoietic cells in mice has been correlated with overactivation of STAT3 in response to IL-6 and led to development of neutrophilia and general inflammation [116].

SOCS3 is mainly transcribed by STAT1 and STAT3 [117,118], although other transcription factor like Sp3 have been shown to play a role [119,120] via binding to a GC-rich motive in proximity of the TATA-box on the promoter [121]. Identical to SOCS1, SOCS3 transcription is activated by CpG methylation within the promoter. Under stress conditions, for example upon viral infection, SOCS3 mRNA can be translated from different starting codon increasing the translation level of the protein and its stability, without compromising its activity [122]. The stability of the protein is in addition mediated by the SOCS box. Modulation of the half-life of SOCS3 has been shown to be dependent on the binding of ubiquitin machinery to the SOCS box with considerable extension of the half-life of the protein through stabilization of the complex [110,123,124]. Moreover SOCS3 is the only SOCS protein containing a 35 amino acid PEST [125] (Proline (P), Glutamate (E), Aspartate (D), Serine (S) and Threonine (T)) motive which has been described to be recognized as a site for protein degradation [126] and has been found to be responsible for the cellular

turnover of this protein. In fact, removing PEST drastically increased the half-life of SOCS3 without compromising its activity [125].

3.2.3.3 CIS and SOCS2

CIS and SOCS2 have a similar function as they both target STAT5, although not exclusively. CIS has been shown to bind to the STAT5 binding sites of the receptors and thus inhibiting the activation of the transcription factor. CIS is involved in the ubiquitination process and consequent degradation of the EPO receptor [127].
SOCS2 was found to bind to the GH receptor inhibiting the binding and activation of STAT5β [128]. SOCS2 knock out mice showed increased body weight suggesting the inability of these mice to control GH signaling [129-131].

3.2.3.4 Other SOCSs

Not much is known about the remaining SOCS family members. Biological function of SOCS4, SOCS5, SOCS6 and SOCS7 remains poorly defined. SOCS4 and SOCS5 have been found to be expressed at high levels upon stimulation with EGF. SOCS5 upregulation correlates with EGF receptor degradation [132]. SOCS5 is induced via IL-6 signaling [133]. Knock-out mice for SOCS6 have a mild growth defect implying a possible role for SOCS6 in the control of GH signaling [134]. SOCS6 and SOCS7 were found to bind regulatory subunits of phosphatidylinoitol 3 kinase pathways with a possible role in the control of insulin signaling [134,135].

3.2.4 Role in viral infection

The role of SOCS proteins in the context of viral infection is an emerging field [74]. A number of viruses have been found to upregulate these proteins, especially SOCS1 and SOCS3, to their advantage (listed in Table 3.2) [74,136-151]. Modulation of SOCS proteins correlate with an enhanced infectivity and replication capacity mainly by attenuating the activation and the production of type I and II IFNs [74]. The IFNs represent a primordial JAK/STAT dependent, tightly regulated defense system against acute viral infection and belong to one of the most important antiviral cytokine families (Figure 3.3) [152].
Two members of the herpesvirus family exploit these proteins: Herpes simplex virus 1 (HSV-1) has been found to upregulate SOCS1 and SOCS3 to counteract type I

and type II IFN signaling [140-143]. Interestingly the induction of SOCS1 and SOCS3 is highly cell specific. After HSV-1 infection, SOCS1 has been found to be upregulated in keratinocytes but not in fibroblast. SOCS1 was found to be essential for the control of the type II IFN pathway [140]. In cells that upregulate SOCS3 expression upon HSV-1 infection, silencing of the protein led to a reduction of viral progeny titer produced, thus reducing the replication efficiency [142]. The ability of the virus to escape type I IFN cell response has been linked to the upregulation of SOCS3 [141].

Table 3.2: Viruses known to exploit SOCS for their advantage (adapted from ([74])
Abbreviation: CT-1, cardiotrophin-1; other explained in the text and in the abbreviation list.

Virus	SOCS protein	Function[a]
Coxsackievirus	SOCS1	Inhibits IFN-α, IFN-β, and CT-1 signaling
	SOCS3	Inhibits CT-1 signaling
Ebola virus	SOCS1	May ubiquitinate VP40 to enhance progeny virus production
HBV	SOCS1	Inhibits IFN-α production
	SOCS3	Inhibits IFN-α production
HCV	SOCS1	Increased expression in T cells decreases T cell activation and IFN-γ production; decreased expression in B cells increases B cell activation, proliferation, and Ab production
	SOCS3	Inhibits IFN-α signaling
HSV-1	SOCS1	Inhibits IFN-γ signaling
	SOCS3	Inhibits IFN-α and IFN-β signaling and IFN-α production
HIV-1	SOCS1	Ubiquitinates HIV-1 Gag to enhance progeny virus production
	SOCS1/SOCS3	Inhibits IL-4 to prevent Ab class switching; inhibits IL-10 to prevent Ab production
	SOCS2	Inhibits IFN-γ signaling
	SOCS3	Inhibits IFN-β signaling
Influenza virus	SOCS3	Inhibits IFN-β signaling
RSV	CIS	Inhibits IFN-α signaling
	SOCS1	Inhibits IFN-α signaling
	SOCS3	Inhibits IFN-α signaling

A further member of the herpesvirus family, Epstein Barr virus (EBV), upregulates SOCS3 to its advantage. EBV was first described to upregulate mRNA of SOCS1 and SOCS3 in infected cells, but no mechanism involving SOCS was assessed [153]. Recently, primary monocytes infected with EBV were demonstrated to upregulate SOCS3 and to lesser extent SOCS1 mRNA and proteins. The EBV protein Zta activated SOCS3 expression which led to the suppression of IFNα secretion and favored a state of type I IFN irresponsiveness by downregulating STAT2 phosphorylation [139].

To date only two studies have shown a link between modulation of SOCS and HCMV. These studies were carried out in monocytes or monocyte-derived dendritic cells (MoDCs). Both investigations showed upregulation of SOCS3 correlated with the control of cytokine expression [154,155]. The first study was carried out in monocytes and focused on the ability of cmvIL-10 (a homologous to human IL-10) to

suppress cytokine secretion of cells. Cells were not infected but stimulated with cmvIL-10. This report showed that cmvIL-10 inhibits NF-κB activation by reducing the degradation of NF-κB inhibitor IκB-α and consequently reduced the expression of NF-κB transcribed genes (e.g. TNFα, IL-1β). In this context an upregulation of SOCS3 mRNA upon human IL-10 stimulation was detected (which was already published [156-158]), whereas protein modulation was not assessed. Upregulation of SOCS3 mRNA was not directly linked to NF-κB inhibition. The authors pointed out the ability of cmvIL-10 to induce expression of negative immunoregulators like SOCS3 [154].

More recently, in late 2011, a publication on MoDCs and CMV showed that CMV infection of MoDCs (CMV-MoDCs) resulted in mRNA upregulation of SOCS1 and SOCS3. Expression of SOCS3 was linked to IL-6 stimulation. It was reported that the GM-CSF signaling was impaired in the whole population of CMV-MoDCs, although only half of the cells were productively infected. IL-6-induced SOCS3 was at least partially responsible for blocking GM-CSF mediated STAT5 signaling in CMV-MoDCs since silencing of SOCS3 lead to the rescue of STAT5 phosphorylation. SOCS1 was not involved in this process [155].

In both reports SOCS3 was claimed to be at least partially involved in HCMV mediated mechanism of immunosuppression but no direct link between SOCS3 and replication ability of HCMV was provided [154,155].

3.2.5 Porcine SOCSs

Porcine SOCS genes and proteins are poorly characterized so far. It is known that all the 8 components of the SOCS protein family (CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7) are expressed as mRNA at a constitutive level in porcine tissues. Interestingly, SOCS1, SOCS3, SOCS7 and CIS were found to be expressed at variable constitutive levels in the different tissues analyzed, whereas for SOCS2, SOCS4, SOCS5 and SOCS6 the expression levels were less variable [159]. Expression of porcine SOCS1 was found to be increased upon infection or vaccination against porcine reproductive and respiratory syndrome virus [160,161]. Infection with *Salmonella enterica, Serovar choleraesuis* and *typhimurium* increased expression of porcine SOCS1 [162]. Porcine SOCS2 has been cloned [163] and its expression was induced in the epithelium in response to *Entamoeba histolytica* [164]. Porcine SOCS3 mRNA expression was increased by butyrate [165] and decreased

by *Pasteurella multocida* toxin resulting in an constitutive activation of STATs [166]. SOCS3 expression in muscle and adipose porcine tissues was found to be related to obesity via control of activation of insulin-like growth factor I, which is involved in both muscle development and adipose metabolism. The different SOCS3 transcription profiles between obese and lean pig in muscle and adipose tissue were correlated with the different fat depositions and muscle development [167,168].

3.3 Aim of the study

SOCS proteins are powerful controllers of JAK/STAT related pathways. These proteins are exploited by numerous viruses to control the immunoresponse to their advantage.

We hypothesized that interference of HCMV with SOCS proteins results in an attenuation of the cellular immune response to the advantage of HCMV.

To test this hypothesis in our endothelial model of HCMV infection a step-wise experimental approach was designed:

- Examination of SOCS1 or SOCS3 modulation upon HCMV infection, on the mRNA and protein level.
- Silencing of SOCS1 and SOCS3 to evaluate the impact on the replication efficiency of HCMV.
- Investigation and elucidation of potential molecular mechanisms underlying the HCMV-SOCS interaction.

4. Results part I

This part is summarizing the development of the thesis in a chronological order. It includes unpublished results, problems and troubleshooting that have arisen during the work and all the steps that finally led to the results included in the manuscript and presented in results part II (Chapter 5).

Material and methods are included in the chapter 5, section 5.6. Other protocols not described in section 5.6 are included in section 4.3.

4.1 Porcine setting

This part of the project was based on a previous thesis carried out in our Lab by Maddalena Ghielmetti [169]. She established a real time polymerase chain reaction (RT-PCR) multiplex system specific for porcine SOCS1 or SOCS3, which was used to measure mRNA expressions.

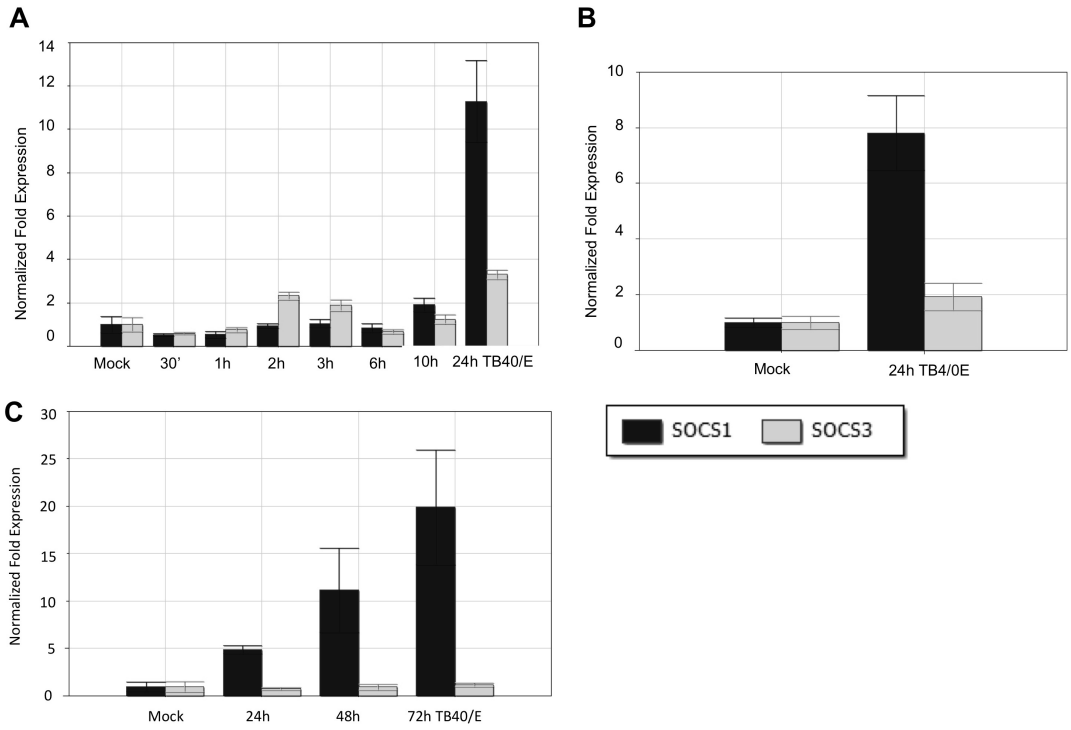

Figure 4.1: SOCS1 mRNA expression is upregulated by HCMV infection in primary PAEC-KO as from 24 hours p.i.

Time course analysis of TB40/E-infected PAEC-KO (multiplicity of infection (MOI) of 1) show an upregulation of SOCS1 (black) transcription starting 24 hours p.i. (A, B and C). In contrast SOCS3 (gray) mRNA expression was not modulated upon infection. (Mean with standard deviation (SD) of one representative experiment carried out in duplicate (A and C), Mean of two independent experiments (B). Results are related to the non-infected control (mock) and are normalized to GAPDH expression.

4.1.1 Detection and modulation of SOCS1 and SOCS3 in HCMV-infected porcine cells

HCMV productively infects porcine endothelial cells [26,27]. It is expected that after xenotransplantation of porcine grafts into human recipients, HCMV is reactivated due to the immunosuppression needed. SOCS1 and SOCS3 are strong negative

modulator of cytokine pathways that are known to play an important role in rejection process (i.e. IFNγ; IL-6). Based on the RT-PCR system established in our lab [169] we investigated the expression of SOCS1 and SOCS3 transcripts in HCMV infected pECs.

We first carried out a time course with primary porcine aortic endothelial cells knock-out for α1,3 galactosyltransferase (PAEC-KO, Figure 4.1A). This analysis showed a 10 times higher expression of SOCS1 mRNA 24 hours p.i. as compared to non-infected (mock) PAEC-KO (Figure 4.1A and 4.1B). SOCS1 transcription in HCMV infected PAEC-KO increased during the course of infection as shown in figure 4.1C, whereas no modulation of SOCS3 mRNA transcription was observed.

Other porcine endothelial cell lines were analyzed for HCMV mediated modulation of SOCS1 or SOCS3 mRNA. The time course experiments (same time points as figure 4.1A and C) did not show any clear modulation of SOCS1 or 3 transcripts in both 2A2 (bone marrow derived endothelial cells) or PEDSV.15 (endothelial cells from aortic origin) [170]. Reanalysis of one time point are shown in figure 4.2 were the mean of two independent experiments indicates no modulation of SOCS1 or SOCS3 transcription 24 hours p.i..

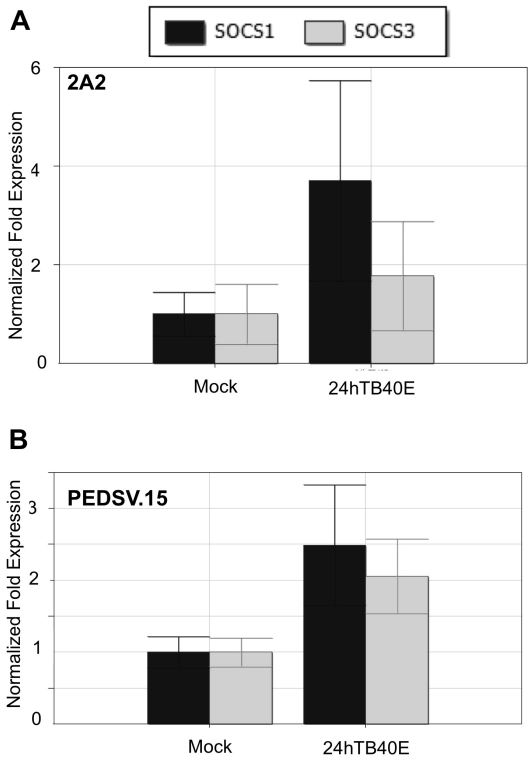

Figure 4.2: Cell-specific modulation of SOCS1 and SOCS3 in HCMV-infected porcine endothelial cell lines

2A2 (A) and PEDSV.15 (B) immortalized porcine endothelial cells were infected for 24h with TB40/E at an MOI of 1. Analysis of SOCS1 (black) and SOCS3 (gray) mRNA expression showed no significant viral mediated modulation. Shown are the means with SD of two independent experiments. Results are related to the non-infected control (mock) and are normalized to GAPDH expression.

Based on the results obtained with HCMV-infected PAEC-KO on the mRNA level proteins were analyzed by western blot. Figure 4.3 shows SOCS1 and SOCS3 protein in infected and stimulated cells respectively. Cross-reactivity of both SOCS1 and SOCS3 antibodies for porcine proteins primarily designed against human proteins was established first. A remarkable upregulation of SOCS3 was detected in cells stimulated with TNFα, Lipopolysaccharide (LPS) or PMA. SOCS1 seemed to be slightly modulated by PMA stimulation although the staining for SOCS1 remained inconsistent rendering interpretation difficult. In summary, HCMV infected PAEC-KO did not show an obvious viral mediated modulation of both SOCS1 and SOCS3 (Figure 4.3).

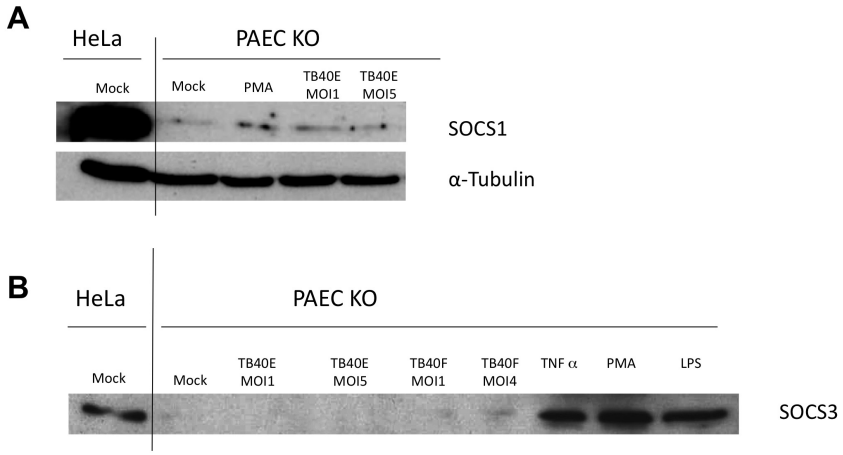

Figure 4.3: Detection of SOCS1 and SOCS3 proteins in PAEC-KO

Western blot analysis of PAEC-KO revealed no modulation of SOCS1 (A) in cells infected with TB40/E (MOI of 1 or 5). Stimulation with PMA (500ng/ml) slightly upregulated SOCS1 expression. SOCS3 (B) was not modulated by the infection (TB40/E MOI of 1 or 5 and TB40/F MOI of 1 or 4). Strong upregulation of SOCS3 was observed if cells were stimulated with PMA (500ng/ml), TNFα (100U/ml) or LPS (100ng/ml). HeLa cells lysate was used as positive control.

4.2 Human setting

The interesting results obtained in the porcine setting prompted us to broaden the range of our research. Since no data were published about a possible interaction between HCMV and SOCS in human cells we decided to focus our efforts on this topic. The project, which was developed initially within a cross-species xenotransplantation context, now included studies of HCMV in cells of the same species.

The results presented in this part are the summary of the results obtained in the human setting and describe the work leading to the submitted manuscript. The paper contains the essence of the findings and is presented in the next chapter (Chapter 5).

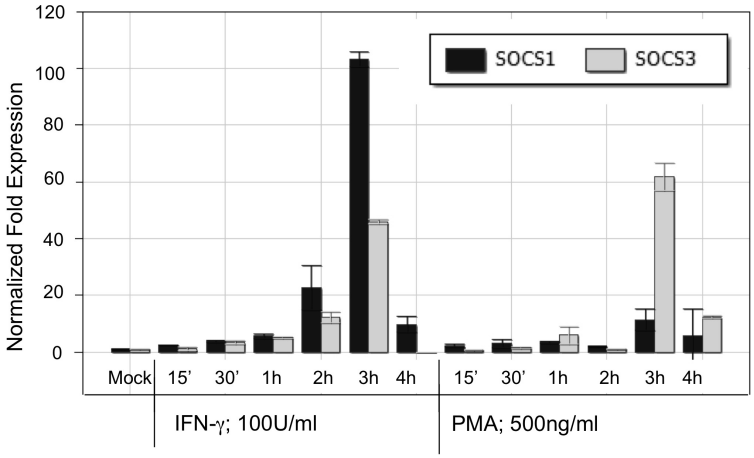

Figure 4.4: Detection and modulation of SOCS1 and SOCS3 mRNA in fibroblast

MRC5 were stimulated with either IFNγ (100U/ml) or PMA (500ng/ml) at the given time points. mRNA expression of SOCS1 (black) and SOCS3 (gray) was upregulated by both stimuli. Results are related to the non-treated control (mock) and normalized to GAPDH expression. Shown is the mean with SD of one representative experiment carried out in duplicate.

4.2.1 Upregulation of SOCS1 and SOCS3 in human cells upon stimulation

To evaluate the expression of SOCS1 and SOCS3 in human cells and to validate the RT-PCR system, human lung derived fibroblast (MRC5) were stimulated with PMA and IFNγ, two inducers of SOCS1 and SOCS3 [93,171]. MRC5 reacted to both stimuli by upregulating the expression of both transcripts reaching the highest level at 3 hours post stimulation (p.s.) for IFNγ with about 100 and 50 times more mRNA

expression of SOCS1 and SOCS3, respectively, as related to the non-stimulated control. With PMA upregulation reached the top level as well at 3 hours p.s. with an increase of about 15 times for SOCS1 and 50 times for SOCS3 (Figure 4.4).
After these first results obtained with MRC5, a cell line with the advantage of rapid growth and uncomplicated handling, the decision was taken to analyze SOCS1 and SOCS3 expression in primary endothelial cells. These cells were chosen for their important anatomical location since they form the first barrier between recipient blood and transplanted graft. They are known natural targets of HCMV infection. We stimulated human primary lung-derived endothelial cells (HMVEC) with IFNβ (Figure 4.5), IFNγ (Figure 5.1A and C, Chapter 5), PMA (Figure 5.1B and D, Chapter 5),

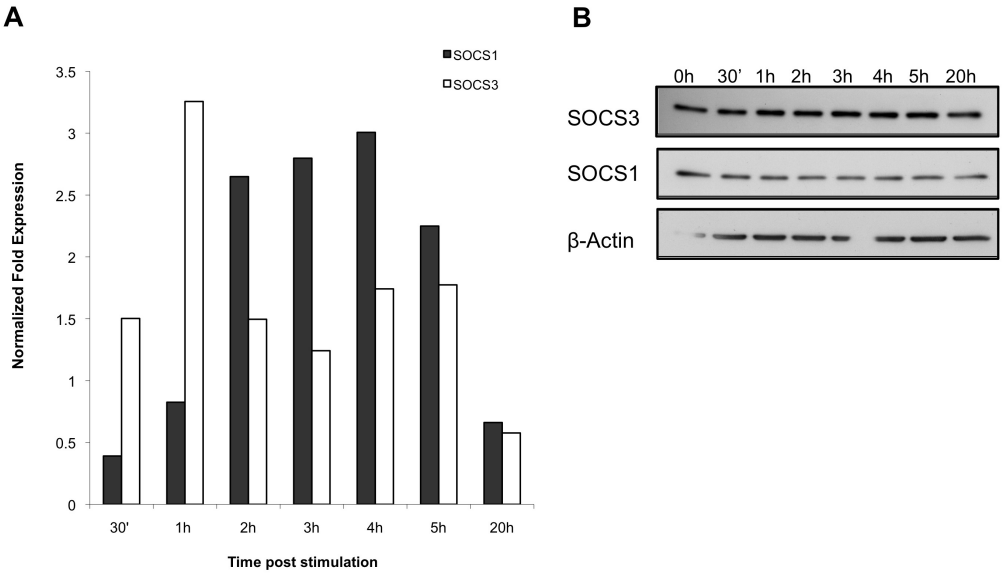

Figure 4.5: Stimulation with IFNβ of HMVEC modulates SOCS1 and SOCS3 expression
Time course analysis of HMVEC stimulated with 100U/ml IFNβ resulted in a slight upregulation of mRNA expression of SOCS1 (black) and SOCS3 (white). mRNA expression levels are related to the untreated control and normalized to GAPDH expression. Shown is one representative experiment (A). SOCS3 protein was upregulated whereas SOCS1 protein did not show any increase (B). 5 hours p.s. cells were washed and incubated in normal medium for 15 more hours showing that SOCS1 and SOCS3 expression is reduced to the constitutive level within 15 hours.

TNFα and LPS. SOCS1 and SOCS3 mRNA were both modulated by IFNβ, IFNγ and PMA whereas no modulation was observed within the first 5 hours of stimulation with

TNFα or LPS (data not shown). Interestingly, although mRNA upregulation of both SOCS1 and SOCS3 with IFNβ was lower than what was observed for IFNγ or PMA, we obtained exactly the same pattern on the protein level, where SOCS3 was in line with mRNA and increased over time. On the contrary, SOCS1 did not show any increase on the protein level or even a slight decrease, despite enhanced mRNA expression (Figure 4.5 and Figure 5.1, Chapter 5).

4.2.2 Detection and modulation of SOCS1 and SOCS3 in HCMV-infected human cells

Establishment of the RT-PCR system with stimulated samples and verification of the expression and modulation of SOCS1 and SOCS3 was followed by a time course analysis of HCMV infection in three different cell types. Primary HMVEC and aortic endothelial cells (HAEC) and immortalized MRC5 were used (Figure 4.6). The results revealed a different pattern of modulation for the three cell types. HMVEC (Figure 4.6A) showed a 12-fold upregulation of SOCS1 at 10 hours p.i. after HCMV infection, whereas SOCS3 was found to be slightly upregulated 2 hours p.i., although SOCS3 modulation was not consistently observed (Figure 4.6A, Figure 5.2, Chapter 5). HAEC (Figure 4.6B) showed an upregulation of SOCS1 already 2 hours p.i., which was weaker then the one observed for HMVEC and much less reproducible. SOCS3 did not show any remarkably modulation. In MRC5 (Figure 4.6C) neither SOCS1 nor SOCS3 were strongly modulated. Based on these results and on other similar experiments we decided to further investigate the role of HCMV in HMVEC. Reproduction of the time course analysis of HCMV infected HMVEC consistently led to an upregulation of SOCS1 mRNA between 7 and 12 hours p.i. and these primary endothelial cells were the most relevant for our model.

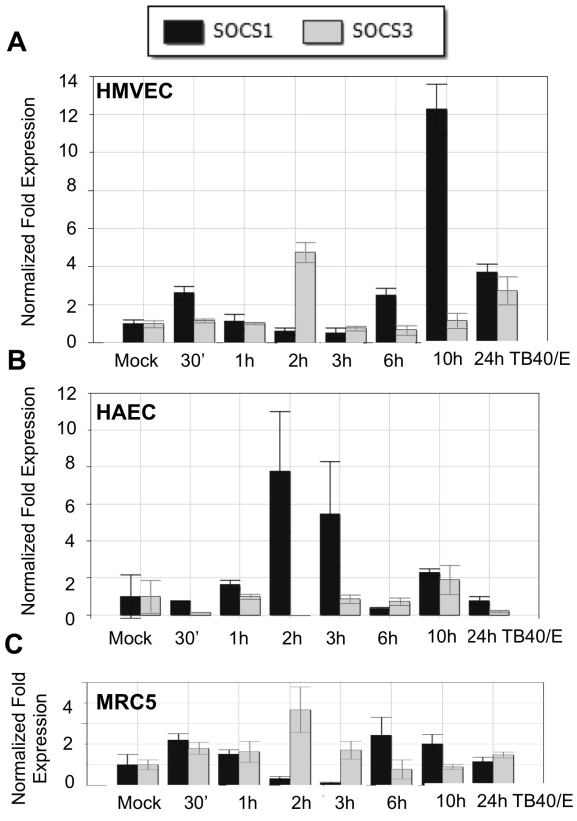

Figure 4.6: Time course expression of SOCS1 and SOCS3 mRNA in HCMV-infected human cells

Time course analysis of HMVEC (A), HAEC (B) or MRC5 (C) infected with HCMV showed upregulation of SOCS1 (black) mRNA for the endothelial cells at 10 hours p.i. for HMVEC and at 2-3 hours p.i for HAEC, with a maximum 12-fold and 8-fold increase, respectively. In MRC5 infection did not induce any strong modulation of the transcripts. SOCS3 (gray) was not modulated by infection in none of the cells tested. mRNA expression levels are related to the non-infected control (mock) and normalized to GAPDH expression. Shown is the mean with SD of one representative experiment carried out in duplicate.

4.2.3 Silencing of SOCS1 and SOCS3 in HMVEC leads to impaired HCMV replication

Silencing of primary cells has always been a challenge due to their refractory nature to transfection and their fragility. HMVEC were transfected with short interfering RNA (siRNA) targeting SOCS1 (siSOCS1), SOCS3 (siSOCS3) or a scramble non-specific control (siCNTR) (Santa Cruz Biotechnology, Heidelberg, Germany). The magnitude of the silencing was difficult to assess on the protein level. SOCS1 was undetectable for most of the time points in both siSOCS1 and siSOCS3 populations indicating possible cross-reactivity of the siRNAs. Most importantly, infected siSOCS1 and siSOCS3 cultures resulted in lower viral antigen production compared with siCNTR and the non-transfect (N.T.) cultures (Figure 4.7).

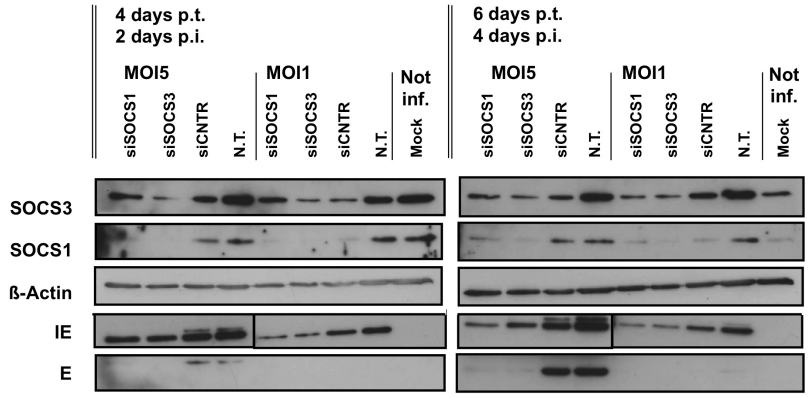

Figure 4.7: Silencing of SOCS1 and SOCS3 lead to lower viral antigen production in HCMV infected HMVEC

Silencing of SOCS1 and SOCS3 abrogated IE and E antigen production in HMVEC for both MOI of 1 and MOI of 5 inoculums compared to cultures transfected with an unspecific siRNA and N.T. cells. Lysates were collected either 4 days post transfection/2 days post infection (4 days p.t., 2 days p.i.) or 6 days post transfection/4 days post infection (6 days p.t., 4 days p.i).

To evaluate the impact of SOCS1 and SOCS3 knock-down on HCMV replication, titration of the supernatant for the different conditions was carried out. Figure 4.8 showed that silencing of SOCS1 and SOCS3 had a significant effect on the infectious particles produced in these cultures. siSOCS3 virus titer was reduced by 10 fold as compared to the one collected in siCNTR or non transfect cultures. siSOCS1 resulted in a 5 fold decrease for both multiplicity of infection (MOI) of 5 (Figure 4.8A) or MOI of 1 (Figure 4.8B). The effect of silencing is limited by many factors including metabolism of the cells, magnitude of expression of the target genes, stability of the siRNA. Since determination of the silencing rate by western blot was difficult, HMVEC were transfected a second time 4 days after the first transfection to evaluate the impact of a second silencing on viral replication. Analyses of viral antigen production (not shown) were in line with the results found for cultures transfected once (Figure 4.8) despite a tendency of twice transfected siSOCS3 to even further decrease the viral titer. The fact that the double transfection did not increase the effect on the viral replication suggested that the single silencing was still effective 5 days p.i..

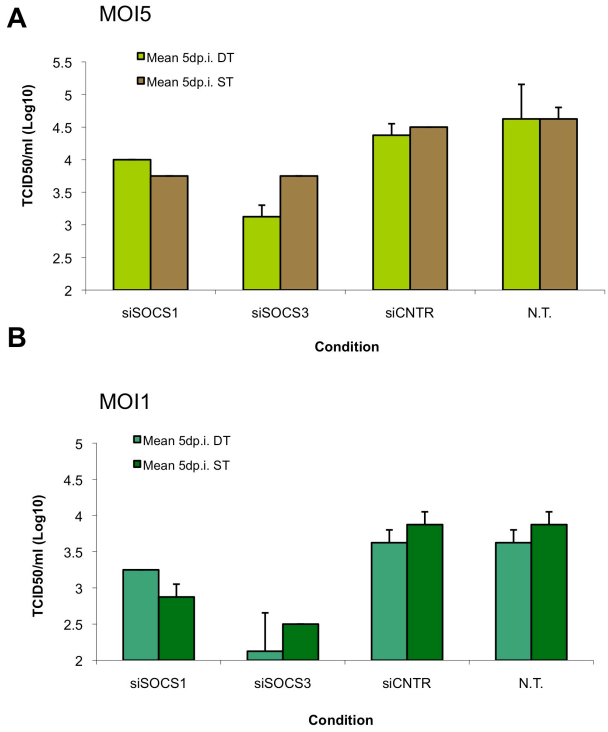

Figure 4.8: Reduced HCMV infectious viral particles production in siSOCS1 and especially siSOCS3 HMVEC

HCMV titer collected in cells infected with MOI of 5 (A) or MOI of 1 (B) was reduced in siSOCS1 and more evidently in siSOCS3 HMVEC. Shown are the results for HMVEC transfected once (single transfection ST) or twice (double transfection DT). In both cases 5 days p.i. the magnitude of the reduction in the titer was about the same, with a tendency of increase in siSOCS3 DT. Shown is the mean with SD of one representative experiment carried out in duplicate.

4.2.4 Optimization of the silencing and western blot technique reveal IFN type I activation in silenced HMVEC

Despite our data indicating a role for SOCS1 and especially for SOCS3 in HCMV replication in HMVEC, the results raised a number of questions. In particular, the issue related to the determination of the magnitude and specificity of the silencing by western blot was not resolved satisfactorily. Optimization of the western blot analyses for SOCS1 and SOCS3 were carried out first by quantification of the cell lysate with a nanodrop instrument. To assess the reliability of this device and

compatibility with our lysis buffer (LB), titration of bovine serum albumin (BSA) diluted with the LB was

carried out. Figure 4.9A shows that despite a difference between the expected concentration (based on the stock concentration of the BSA) and the one measured with the nanodrop instrument the error between the two values was constant in a range from 3 to 0.3μg/μl. Using this technique of quantification we were able to load the same amount of lysate in all the slots of the sodium dodecyl sulfate polyacrylamide gel (SDS-Gel) avoiding variation of intensity of the bands due to slightly different amount of proteins loaded. After establishing the protocols with the nanodrop instrument, the silencing experiments were reanalyzed for SOCS1, SOCS3. The results showed that siSOCS3 led to SOCS3 downregulation but also to a lower SOCS1 expression. siSOCS1 in contrast showed a specific downregulation of the target protein (Figure 4.9B). A crucial additional analysis was performed by measuring Mx expression. Mx is an indicator for the activation of type I IFN pathway which is a very potent natural inhibitor of viral replication. We had to carefully rule out that the observed reduction in HCMV antigen and titer was a mere result of an unspecific antiviral state provoked by silencing. Indeed, analysis of Mx expression revealed an unexpected induction of this protein for both siSOCS1 and siSOCS3 samples (Figure 4.9B).

Abolishing the induction of Mx was mandatory to validate the results collected with the virus and for this aim many different strategies and compound for the transfection were evaluated. All the experiments previously described were carried out following the so-called forward transfection, basically adding the transfection reagent to the monolayer of cells. In figure 4.9C different transfection compounds were tested for reverse transfection, such that the cells were seeded on the transfection solution. Lipofectamin 2000, the compound we usually employed, always gave a strong induction of Mx, whereas with HiPerFect and Lipofectamin RNAiMax, Mx was basically not detected. Unfortunately, the level of silencing detected for the target protein was also reduced (Figure 4.9C). Forward transfection with HiPerFect did not lead to better silencing results and showed induction of Mx (Figure 4.9D).

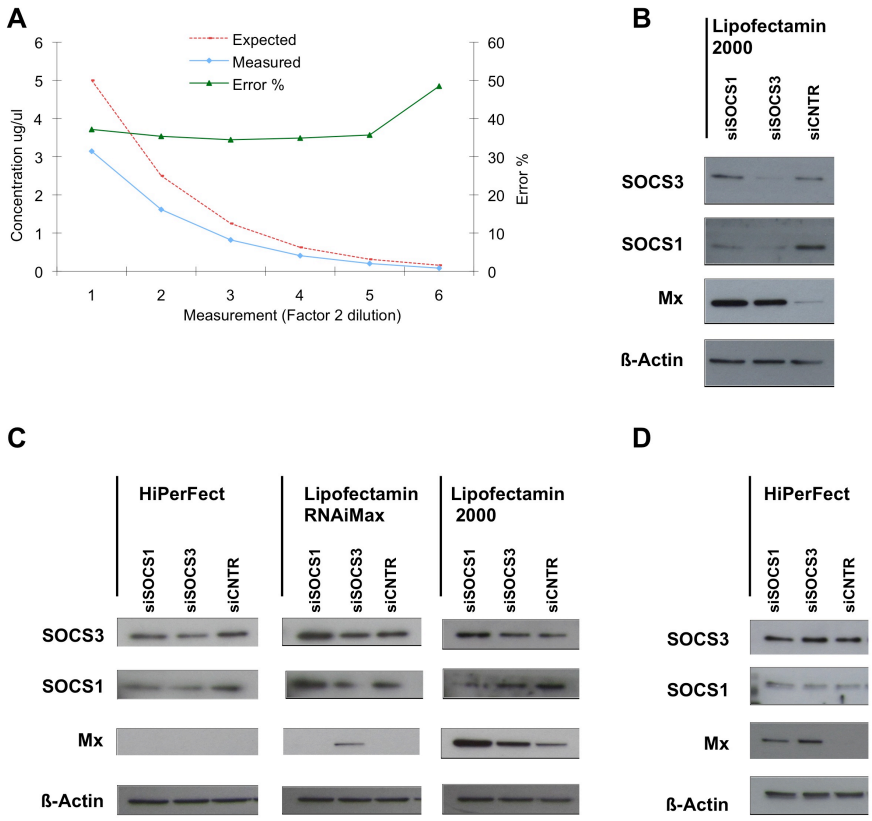

Figure 4.9: Protein quantification, western blot optimization and detection of Mx in transfected HMVEC

(A) Reliability of nanodrop protein quantification was evaluated with a titration of BSA diluted in the usual LB. The expected results (red) based on the stock concentration of the BSA were always higher then the measured results (blue) with an error (green) that was constant until very low protein concentrations. (B) Forward transfection with Lipofectamin 2000 lead to Mx expression. (C) Reverse transfection with different compounds did not show any remarkable silencing for HiPerFect and Lipofectamin RNAiMax; whereas for Lipofectamin 2000 silencing and Mx induction were both detected. Forward transfection with HiPerFect led to Mx induction without increasing the level of silencing.

4.2.5 Establishment of a lentiviral transduction system for efficient delivery of short hairpin RNA targeting SOCS1 and SOCS3

To overcome Mx induction, simultaneously with the different compound and transfection strategies, a lentiviral transduction system for delivery of short hairpin RNA (shRNA) was designed and developed as originally described by [172] and further developed by [173]. This technique offered a number of advantages

compared to normal transfection. First the vector expressing shRNA was integrated into the genome of the transduced cells allowing longer expression of the silencing system. It is much less toxic for the cells than any transfection compound and carried a green fluorescence protein (GFP) coding box allowing the determination of the ratio of transfected cells by fluorescence microscopy. The system is based on three vectors, which were transfected into 293 HEK cells leading to the production of lentiviral particles carrying the shRNA expressing vector with the GFP-box.

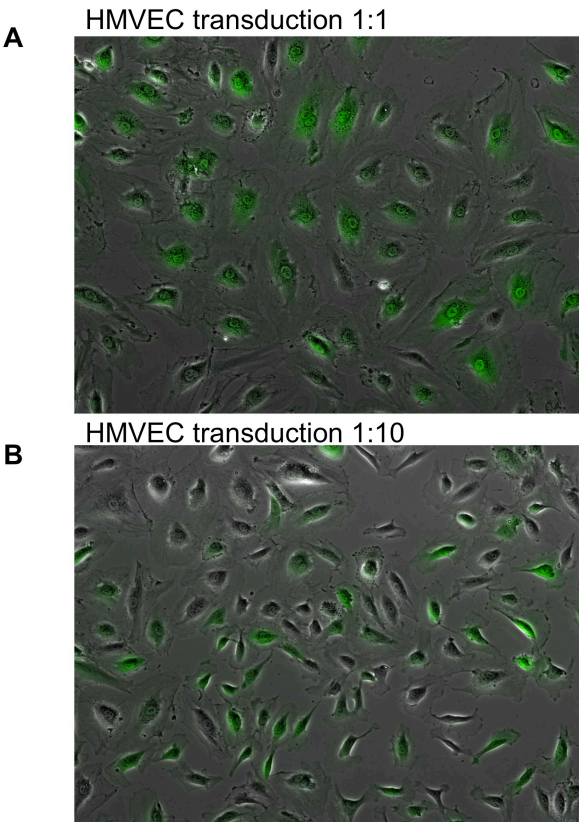

Figure 4.10: Lentiviral transduction of HMVEC

Lentiviral particles were collected from 293 HEK cells and added undiluted (A) or diluted 1 to 10 (B) to HMVEC. Shown are the pictures of the cultures 3 days post transduction, overlay of GFP signal and phase contrast microscope pictures.

Supernatant of 293 HEK cells, containing wild type lentiviral particles was collected and added undiluted or 10-fold diluted to HMVEC to assess the ability of the pseudoviral particles to efficiently transduce this type of cells. Figure 4.10 shows the GFP expression in HMVEC 3 days post transduction demonstrating for the first time that HMVEC are transducible with this lentiviral system. No previous study has shown efficient transduction of HMVEC with this combination of vectors. Two

SOCS1, one SOCS3 and a scramble shRNA were designed targeting regions of the mRNA that had been shown to be well accessible for the silencing machinery. The sequences were cloned into the delivery vector as described in section 4.3.2. After the packaging, HMVEC were finally transduced with the vectors containing the specific sequences targeting SOCS1 or SOCS3.

Analysis by western blot (Figure 4.11) unfortunately showed no specific downregulation of the target proteins for none of the designed shRNAs. Mx was expressed by HMVEC 2 days after transduction and in a reduced amount 6 days post transduction in shSOCS1_127 and shSOCS1_822 only (Figure 4.11). Quantification by titration of the lentiviral particles based on GFP output allowed the optimization of the dilution rate of lentiviral particles stock solution resulting in 80-90% transfection efficiency (based on GFP expression) and no induction of Mx expression.

Despite non-detectable downregulation of the target proteins HCMV infection of shRNA transduced HMVEC were carried out. As expected no modulation of any viral antigen was observed (data not shown).

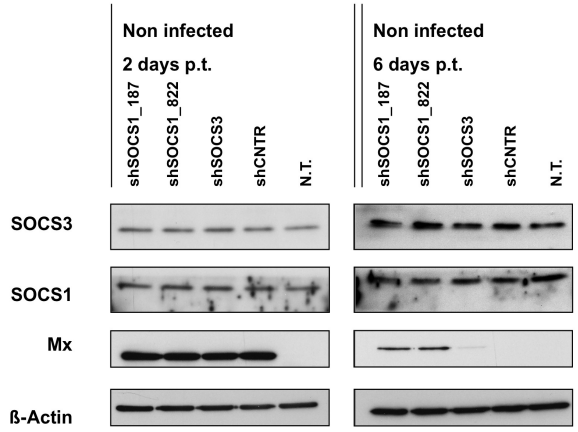

Figure 4.11: Lentiviral transduction of shRNA targeting SOCS1 or SOCS3 does not show any specific downregulation

HMVEC were transduced with shSOCS1_187, shSOCS1_822, shSOCS3, unspecific shRNA sequence (shCNTR) or not transduced. Results 2 and 6 days post transduction show no specific downregulation of the target protein. Mx was detected 2 days post transduction in remarkable amount whereas 6 days post transduction only a weak Mx signal was observed in cultures transfected with shSOCS1_187 and shSOCS1_822.

4.2.6 Silencing of the target protein without Mx induction

Finally, new siRNA were purchased form a different company (Invitrogen) and transfection were carried out with Lipofectamin RNAiMax, the transfection reagent that, based on our experiments appeared to have the best transfection efficiency with the lowest toxicity for HMVEC. With this combination we were able to avoid Mx induction with remarkable downregulation of the target proteins (Figure 4.12 and Figure 5.3A, Chapter 5). In addition the specificity of siRNA appeared to be higher than what had been observed for earlier siRNA. This setting was used for all the silencing experiment included in the Chapter 5.

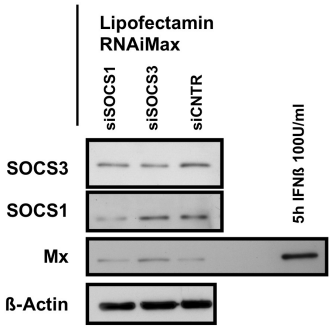

Figure 4.12: Silencing of SOCS1 and SOCS3 without activation of type I IFN pathway
HMVEC were transfected with Lipofectamin RNAiMax and siRNA from Invitrogen targeting SOCS1 or SOCS3. The western blot shows specific downregulation of the target proteins and basically no induction of Mx protein.

4.3 Additional material and methods

Material and methods mentioned in the results part II (Chapter 5, section 5.6) are not listed here.

4.3.1 Real Time PCR system for the porcine setting

RNA extraction and reverse transcription were performed as described in the results part II section 5.6. A real time multiplex system was employed [169] to investigate the expression of SOCS1 (Fw. 5'-GGAACTGCTTCTTCGCCCTC-3'; Rv. 5'-GAAGCGGCCGGCCTGG-3', probe 5'-FAM-TTGTGGGGCCCGAAGCCAT-BHQ1-3'), SOCS3 (Fw. 5'-CCAACGTGGCCACTCTC-3', Rv. 5'-

AGCTGGGTGACTTTCTCATAGGAGTC-3', probe 5'-FAM-AACAGTCAACGGCCACCTG-BHQ1- 3') and GAPDH as housekeeping gene (Fw. 5'-ACATGGCCTCCAAGGAGTAAGA-3', Rv. 5'-GATCGAGTTGGGGCTGTGACT-3', probe HEX-CCACCAACCCCAGCAAGAGCACGC-BHQ1). Primers and probes were mixed in a multiplex powermix (Biorad) for the detection by RT-PCR.

4.3.2 Lentiviral transduction system

Lentiviral system [173] was used to deliver shRNA constructs into HMVEC. shRNA sequences were self designed and obtained from Microsynth AG (Balgach, Switzerland) and are listed below:

shRNA SOCS1-187bp-N-term
….Mlu1……Loop……Stop……NdeI..Cla1
5'-CGCGTccccTGCAGTCTCCACAGCAGCAttcaagagaTGCTGCTGTGGAGACTGCAttttggaaCATATGAT-3'
3'-.....AggggACGTCAGAGGTGTCGTCGTaagttctctACGACGACACCTCTGACGTaaaaaccttGTATACTAGC-5'

shRNA SOCS1-822bp-C-term
….Mlu1……Loop……Stop…NdeI..Cla1
5'-CGCGTccccCTGGGATGCCGTGTTATTTttcaagagaAAATAACACGGCATCCCAGttttggaaCATATGAT-3'
3'-.....AggggGACCCTACGGCACAATAAAaagttctctTTTATTGTGCCGTAGGGTCaaaaaccttGTATACTAGC-5'

shRNA SOCS3-2648bp
….Mlu1……Loop……Stop…NdeI..Cla1
5'-CGCGTccccGTCAAACTTTGCACATATTttcaagagaAATATGTGCAAAGTTTGACttttggaaCATATGAT-3'
3'-.....AggggCAGTTTGAAACGTGTATAAaagttctctTTATACACGTTTCAAACTGaaaaaccttGTATACTAGC-5'

shRNA CNTR
….Mlu1……Loop……Stop…NdeI..Cla1
5'-CGCGTccccAGGTAGTGTAATCGCCTTGttcaagagaCAAGGCGATTACACTACCTttttggaaCATATGAT-3'
3'-.....AggggTCCATCACATTAGCGGAACaagttctctGTTCCGCTAATGTGATGGAaaaaaccttGTATACTAGC-5'

After the cloning of the four constructs into the delivery vector (pLVTHM) via the two restriction sides MluI and ClaI Polyethylenimine-mediated (jetPRIME™, Polyplus-transfection® SA, Illkirch, France) co-transfection of 293HEK cells with Gag/Pol (pcMVΔR8.91), Env (pMD-2), and either shSOCS1_127, shSOCS1_822, shSOCS3 or shCNTR constructs cloned into a GFP coding vector (pLVTHM). The wild type plasmids were obtained from Prof. Dr. Roberto Speck, (University Hospital Zurich, Switzerland). Pseudoviral particles were harvested 48 hours after transfection, transferred to Amnicon Ultra 15 ultracell (0.22 m/100kDa) falcon tubes and filtered by centrifugation 4000g for 15 minutes, and immediately used for transduction of HMVEC.

4.3.3 Additional reagents

For the detection of porcine SOCS by western blot the following antibodies were used: SOCS1 antibody (sc-9021, Santa Cruz Biotechnology) and SOCS3 antibody (#2923 Cell Signaling).

Transfection of HMVEC with siRNA has been tested with HiPerFect (Qiagen), Lipofectamin 2000 (Invitrogen) in both forward (cells are seeded and the transfection reagent was added to the monolayer) or reverse (first transfection reagent to the well and then cells were added) transfection protocol.

5. Results part II

This part includes the manuscript recently submitted. This chapter contains the main results of the thesis.

Suppressor of Cytokine Signaling 3 is a Key Factor for Efficient Cytomegalovirus Replication in Primary Endothelial Cells

Olmo Sonzogni[1], Anne-Laure Millard[1], Aline Marie Taveira[1], Marten K.J. Schneider[2], Li Duo[1], Roberto F. Speck[1], Nicolas J. Mueller[1]

[1]Division of Infectious Diseases and Hospital Epidemiology, University Hospital of Zurich, University of Zurich, Zurich, Switzerland

[2]Laboratory of Vascular Immunology, Division of internal medicine, University Hospital of Zurich, Raemistrasse 100, 8091 Zurich, Switzerland

Short Title: SOCS3, cytomegalovirus, primary endothelial cells

Submitted to Plos Pathogen

5.1 Abstract

Human cytomegalovirus (HCMV) infection in immunocompromised patients is a major cause of morbidity and mortality. Suppressor of cytokine signaling (SOCS) proteins are very potent negative regulators of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways and have been described to be exploited by a number of viruses facilitating infection and replication by attenuating the immune response of the infected cells. The data presented show that efficient replication of HCMV in primary endothelial cells is depended on SOCS3 protein. Time course analysis revealed an accumulation of SOCS3 in infected cells. Silencing of SOCS3 resulted in a partially inhibited production of immediate early, early and late viral antigens. Consistently, HCMV titer produced by SOCS3 silenced (siSOCS3) cultures were significantly lower as compared to SOCS1 silenced (siSOCS1) or control transfected cultures (siCNTR). Impairment of the replication process due to SOCS3 silencing occurred after entry since the number of infected cells was identical in all three siSOCS1, siSOCS3 or siCNTR cultures. STAT1 phosphorylation was increased in siSOCS3 and siSOCS1 infected cells when compared to siCNTR-treated cells. In contrast, phosphorylation of STAT2 was only increased in siSOCS3 cultures. Analysis by flow cytometry of the ability of the virus to control IFNβ-inducible gene expression showed that the HCMV-infected fraction of siSOCS3 culture had a higher percentage of cells expressing IFNβ-induced MHC-I molecules compared with siSOCS1 or siCNTR. Thus, these findings demonstrate that SOCS3 plays a critical role for efficient HCMV replication in primary endothelial cells and provide evidence for an involvement of SOCS3 in HCMV-mediated control of the type I IFN pathway.

5.2 Author summary

Human cytomegalovirus (HCMV) exploits many avenues to evade the immune response. Here we present data showing for the first time that efficient replication of this virus requires a specific protein called suppressor of cytokine signaling 3 (SOCS3). This protein is responsible for the attenuation of the cytokine-mediated immune response of cells, acting as negative regulator in some of the key pathways involved in this process. A number of viruses have already been shown to use members of the SOCS family to their advantage. Our data demonstrate that in cells with reduced SOCS3 protein expression, HCMV replication is impaired at multiple steps resulting in a marked reduction of infectious progeny. We also provide evidence that SOCS3 plays a critical role in the ability of HCMV to interfere with the immune response. These findings may open new avenues using SOCS3 as a possible target aimed at controlling HCMV replication.

5.3 Introduction

Suppressor of cytokine signaling (SOCS) proteins are very potent negative regulators of the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways. Eight members of this family have been reported, SOCS1 to 7 and the cytokine-inducible SH2 domain-containing protein (CIS) [65-67,83]. SOCS proteins play an important role in maintaining and restoring homeostasis upon cytokines stimulation [75]. They consist of three main domains: the N-terminal which is variable in length and sequence; a central SH2 domain and a C-terminal SOCS box domain [83]. The SH2 domain binds to the phosphorylated target proteins whereas the SOCS box binds to the ubiquitin machinery allowing for poliubiquitination and consequently degradation of the target proteins in the proteasome. SOCS1 and SOCS3 also contain a kinase inhibitor region which can directly inhibit the kinase activity of the target proteins [74,75].

The interferons (IFNs) represent a JAK/STAT-depended tightly regulated defense system against viral infections [152]. SOCS1 and SOCS3 have been investigated for their particular ability to attenuate IFN-mediated responses to viral infections [174]. A number of viruses exploit SOCS1 and SOCS3 [74,136-143,145-151,175], including members of the *herpesviridae* like Herpes simplex-1 virus (HSV-1) [13-16] and Epstein Barr virus (EBV) [12]. Modulation of SOCS1 and SOCS3 proteins correlate with an enhanced infectivity and replication capacity mainly by attenuating the activation and the production of type I and II IFNs [74].

Human cytomegalovirus (HCMV) is a member of the *herpesviridae* family. In immunocompetent adults the infection is asymptomatic but leads to life-long latency and seroprevalence in adults reaches 80%. In immunocompromised patients, such as neonates or after transplantation, primary infection or reactivation of this virus is a major cause of morbidity and mortality [1].

HCMV interference with type I and II IFN activation as a means of immune evasion occurs at different stages. The observed effects are highly dependent on the virus strain and on the cell type of the model investigated [9]. In the well-characterized model of fibroblasts infected with the HCMV Towne strain interference of type II IFN responses occurs at different levels after infection, starting with the inhibition of class II transactivator expression about 6 hours post infection (p.i.) [36]. This is followed by the inhibition of STAT1 phosphorylation by src homology region 2 domain-containing phosphatase, at about 16 hours p.i. [37], and finally by JAK1 disruption 72 hours p.i.

[38]. Downregulation of IFNγ-induced major histocompatibility complex class II (MHC-II) expression in HCMV infected cells has also been demonstrated [36,38-41]. As JAK1 is a shared component of type I and type II IFN pathways the disruption of this protein affects both pathways. Focusing on type I IFN it was additionally shown that p48, another key component of this pathway, was targeted in HCMV infected cells. [42]. Moreover, HCMV has been found to inhibit type I IFN dependent expression of MHC class I (MHC-I), IFN regulatory-1, myxovirus resistance A and 2,5-oligoadenylate synthetase gene expression in fibroblast and human umbilical vein endothelial cells (HUVEC) [9,42], although some of these findings are conflicting as others show an upregulation of these genes in HCMV infected fibroblasts [43-49]. A decrease in the STAT2 phosphorylation followed by a reduction of the total amount of the proteins was recently shown in HCMV infected fibroblast. Degradation of STAT2 was found to be strain dependent and not observed for Towne virus [50]. HCMV-mediated downregulation of MHC-I and MHC-II has been correlated with the ability of the virus to establish latency and escape CD4+ and CD8+ T-cells mediated clearance [36,176-179]. Most of the studies published so far on HCMV infection of adherent cells were carried out in fibroblasts, or HUVEC. For our studies we chose primary human lung-derived microvascular endothelial cells (HMVEC). Being at the interface between blood and tissue the endothelium has been shown to be a strategic natural site of infection for HCMV *in vivo* following a primary infection [29-33] and it is believed to act as viral reservoir [34] involved in viral spread and persistence. Virus-mediated changes in the phenotype of infected endothelial cells may contribute to the dissemination of the virus into organs and has been associated with viral pathogenicity. [35].

To date only two studies have shown modulation of SOCS3 upon HCMV infection of monocytes or dendritic cells and this upregulation was correlated in both cases with control of cytokine expression [154,155]. We investigated the role of SOCS1 and SOCS3 proteins during HCMV infection of HMVEC. Analyzing HCMV-infected HMVEC we show for the first time, that efficient HCMV replication is dependent on SOCS3. We present evidence for a role of SOCS3 in HCMV-induced inhibition of STAT2 phosphorylation and HCMV-mediated attenuation of type I IFN-induced expression of MHC-I molecules, implicating that SOCS3 mediates control of type I IFN pathway activation in HCMV-infected endothelial cells.

5.4 Results

5.4.1 Detection and modulation of SOCS1 and SOCS3 in HMVEC

In unstimulated HMVEC, low levels of SOCS1 and SOCS3 mRNA were detected by RT-PCR. Stimulation with IFNγ, a strong inducer of SOCS1 and SOCS3 expression, increased mRNA transcription reaching the highest levels 5 hours post stimulation (p.s.) for SOCS1 and one hour p.s. for SOCS3 (Figure 5.1A). Analysis by western blot revealed a parallel increase for SOCS3 protein, whereas no modulation of SOCS1 protein was observed (Figure 5.1C). Even when phorbol 12-myristate 13-acetate (PMA) was used instead of IFNγ, the same pattern was observed despite very high mRNA expression levels for SOCS1 (Figure 5.1B and D). For both IFNγ and PMA, after removing the stimulus and keeping the cells for 20 more hours in normal medium, expression of SOCS1 and SOCS3 mRNA returned to levels observed before stimulation, as did SOCS3 protein.

5.4.2 SOCS1 and SOCS3 are modulated by HCMV infection of HMVEC

Time course analysis of SOCS1 and SOCS33 mRNA expression in HCMV infected HMVEC was carried out in cells infected with either a multiplicity of infection (MOI) of 1 or 5. Transcription of SOCS1 was induced within 8 and 12 hours p.i. in a MOI-dependent manner (Figure 5.2A and B). SOCS3 transcription levels, in contrast, were not modulated by neither MOI.

Analysis of SOCS1 and SOCS3 protein levels in HCMV infected HMVEC revealed an accumulation of SOCS3 protein starting between 5-10 hours p.i. on (Figure 5.2C), whereas for SOCS1 no infection dependent modulation of the protein level was observed. The accumulation of SOCS3 was dependent on active viral replication since it was not observed when cells were either infected with UV-inactivated virus (Figure 5.2D) or not infected (Figure 5.2E). Accumulation of SOCS3 was particularly prominent in the late phase of the infection.

These results demonstrate a modulation of SOCS1 and SOCS3 in HCMV infected HMVEC and may indicate an active involvement of these cellular factors in the viral replication process.

5.4.3 Silencing of SOCS3 leads to impaired HCMV replication in HMVEC

To investigate the role of SOCS1 and SOCS3 in the context of HCMV infection of HMVEC short interfering RNA (siRNA) targeting SOCS1 (siSOCS1), SOCS3 (siSOCS3) or a scramble, non-specific sequence as negative control (siCNTR), were transfected in HMVEC resulting in a reduction of the target proteins of about 40% related to siCNTR (Figure 5.3A). Analysis of the infected samples collected 2 (Figure 5.3B) or 5 days p.i. (Figure 5.3C) revealed a major inhibition of viral antigen production in siSOCS3 cells. Mainly the early (E) and the late (L) viral proteins were affected by SOCS3 silencing.

Titration of the supernatants collected 5 days p.i. showed a significant reduction in the number of infectious particles produced by siSOCS3 cultures compared with siSOCS1 and siCNTR cultures for both MOI of 1 and MOI of 5 (Figure 5.3D).

These results further point to an active involvement of SOCS3 during HCMV replication in HMVEC. The effect of SOCS3 silencing is more prominent during the late phase of infection. In line with this observation, in cultures transfected just after infection we obtained similar results for both viral antigens production and supernatants viral titers (data not shown).

5.4.4 Silencing of SOCS3 leads to high level of STAT1 and STAT2 phosphorylation in HCMV infected HMVEC

To elucidate the possible mechanisms underlying the impairment of the HCMV infection of HMVEC upon silencing of SOCS3 we investigated the activation pattern of the different infected cultures (siSOCS1, siSOCS3 or siCNTR) 2 (Figure 5.4A) and 5 (Figure 5.4B) days p.i. for both MOI of 1 and MOI of 5. For both time points, phosphorylation of STAT1 was remarkably increased in siSOCS3 and siSOCS1 as compared to the siCNTR cultures. Within the same condition, no differences between cultures infected with an MOI of 1 or MOI of 5 were observed for the phosphorylation of STAT1. In contrast, STAT2 was found to be phosphorylated only in siSOCS3 cultures infected with an MOI of 5 for both 2 and 5 days p.i. with an increase in phosphorylation of 164% and 171% respectively. Phosphorylation of STAT2 in siSOCS1 cultures did not differ from siCNTR cultures (Figure 5.4A and B). These differences in the phosphorylation of STAT1 and STAT2 between siCNTR and siSOCS3 may indicate a possible implication of SOCS3 in the mechanism that allows the virus to control type I and/or type II IFN pathway activation.

5.4.5 No differences in type II IFN inducible gene expression between siSOCS3 and siCNTR infected cultures

To evaluate the ability of HCMV to control type II IFN pathway in siSOCS1, siSOCS3 and siCNTR HMVEC, cells were stimulated 48 hours p.i. for 24 hours with IFNγ. It was previously shown that HCMV can decreases expression of IFNγ-induced MHC-II molecules [36,38,39]. To measure the capability of HCMV to reduce HLA-DRα transcription, mRNA expression of HLA-DRα was quantified in the three different cultures (siSOCS1, siSOCS3 and siCNTR). Infection of the cultures decreased the transcription of HLA-DRα to about the same level in all 3 conditions (Figure 5.5). These results are in line with the literature [36,38,39] and indicate that HCMV can control type II IFN pathway independently from SOCS1 or SOCS3.

5.4.6 HCMV needs SOCS3 to control type I IFN inducible gene expression

To evaluate the ability of HCMV to control type I IFN pathway in siSOCS1, siSOCS3 and siCNTR HMVEC were stimulated 48 hours p.i. for 24 hours with IFNβ. It was previously shown that HCMV can lower the expression of IFNβ-induced MHC-I molecules [42]. Flow cytometry analysis showed that 3 days p.i the fraction of HCMV infected cells was the same in all the three conditions (siSOCS1, siSOCS3 and siCNTR, Figure 5.6A, B and C respectively). Analyzing the infected fraction only, it was found that the percentage of cells expressing MHC-I molecules was reduced to 8% in siCNTR and siSOCS1 cultures whereas it was found to be about the double (15.3%) in siSOCS3 culture (Figure 5.6D).

These results indicate that SOCS3 may be used by HCMV to inhibit the expression of type I IFN-induced MHC-I molecules in HMVEC.

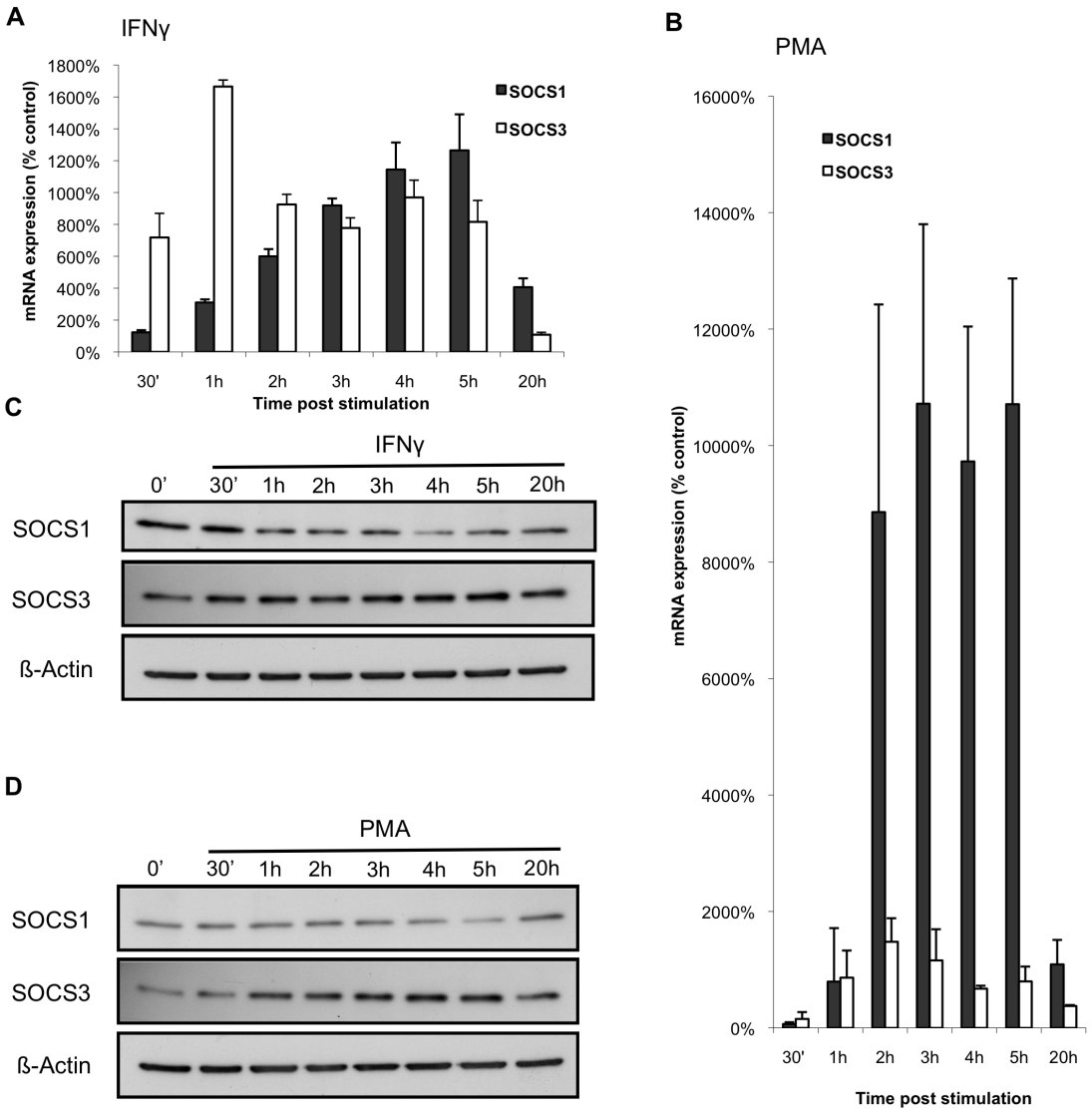

Figure 5.1: Detection and modulation of SOCS1 and SOCS3 in stimulated HMVEC

Time course analysis of HMVEC stimulated with either 100U/ml IFNγ (A and C) or 500ng/ml PMA (B and D) resulted in upregulation of mRNA expression of SOCS1 (black) and SOCS3 (white) in both conditions. mRNA expression levels are given as percentage related to the untreated control (taken as 100%) normalized to GAPDH expression. Shown is the mean with standard deviation (SD) of one representative experiment per condition, carried out in duplicate (A and B). SOCS3 protein was also upregulated by both stimuli whereas SOCS1 protein did not show any increase (C and D). 5 hours p.s. cells were washed and incubated in normal medium for 20 more hours showing that SOCS1 and SOCS3 expression it is reduce to the constitutive level within 20 hours.

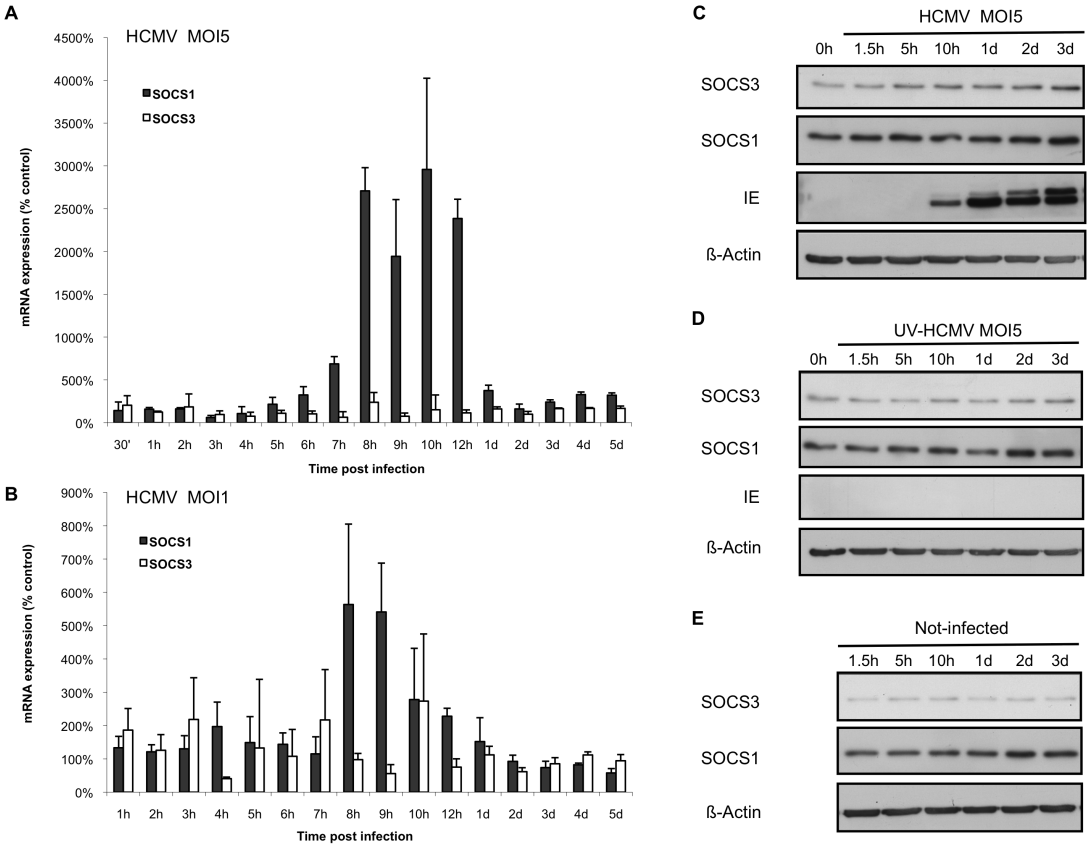

Figure 5.2: HCMV infection of HMVEC induced SOCS1 mRNA expression and SOCS3 protein accumulation

Time course analysis of HCMV-infected HMVEC resulted in an increase of SOCS1 (black) mRNA expression within 8 and 12 hours p.i. for both MOI of 1 or 5, whereas no modulation of SOCS3 (white) was detected. mRNA expression levels are given as percentage related to the non-infected control (taken as 100%) normalized to GAPDH expression. Shown is the mean with SD of one representative experiment, carried out in duplicate (A and B). Western blot analysis of HCMV infected (C), UV inactivated HCMV infect (D) or not infected (E) HMVEC revealed that SOCS3 protein is accumulated only in HCMV infected samples. In contrast, SOCS1 was not modulated.

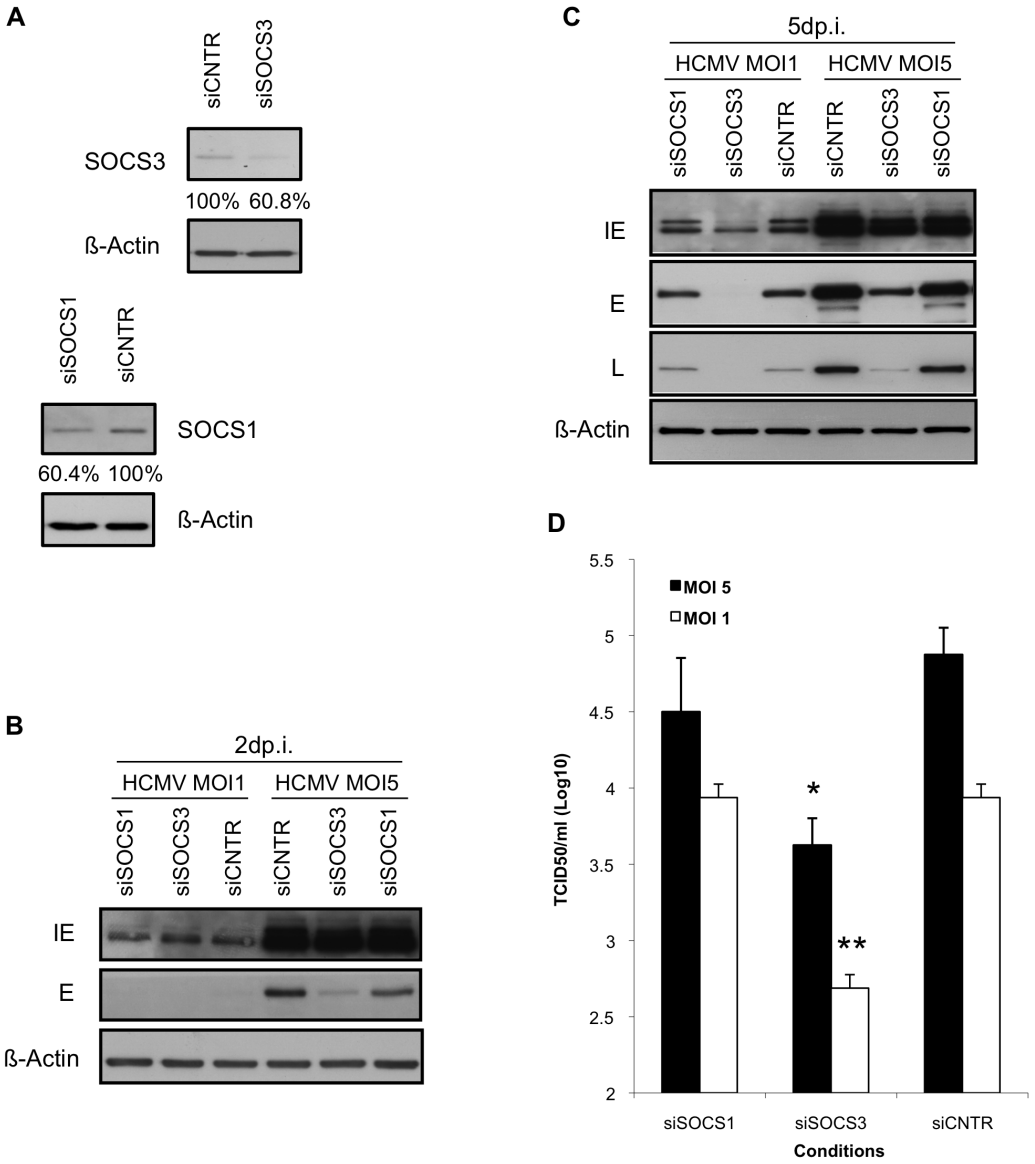

Figure 5.3: HCMV infection of HMVEC is impaired upon silencing of SOCS3

HMVEC were transfected with siRNA targeting SOCS1, SOCS3 and a non-specific control (siSOCS1, siSOCS3 and siCNTR respectively), which resulted in a reduction of target protein expression of about 40% for both siSOCS1 and siSOCS3 with siCNTR taken as 100% (A). After infection with HCMV, siSOCS3 culture showed a reduction in viral antigens (IE, E and L) expression 2 days (B) and 5 days p.i. (C) for both MOI of 1 and 5 as compared to siSOCS1 and siCNTR cultures. The titer of virus produced in siSOCS3 cultures was significantly lower than in siCNTR or siSOCS1 cultures for both MOI of 5 (black) and MOI of 1 (white) inoculums. Shown are the means with SD of two independent experiments, with *$p<0.05$ between siSOCS3 and siCNTR and **$p<0.0001$ between siSOCS3 and both siSOCS1 and siCNTR (D).

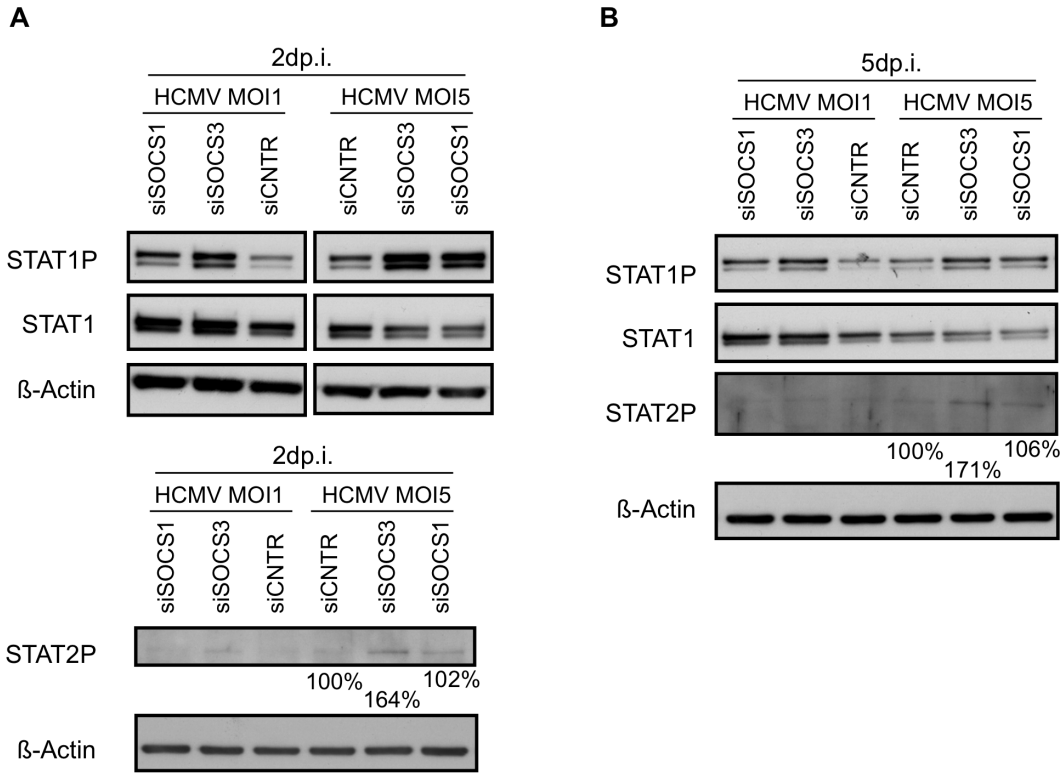

Figure 5.4: Higher phosphorylation of STAT2 in siSOCS3 HCMV-infected HMVEC

HMVEC transfected with siSOCS1, siSOCS3 or siCNTR were infected with HCMV. Phosphorylation of STAT1 was increased in siSOCS1 and siSOCS3 cells 2 (A) and 5 days p.i. (B) for both MOI of 1 and MOI of 5. STAT2 appeared to be phosphorylated only in siSOCS3 cultures infected with MOI of 5 with a level that reached 164% and 171% of siCNTR phosphorylation at day 2 (A) and day 5 (B) p.i., respectively.

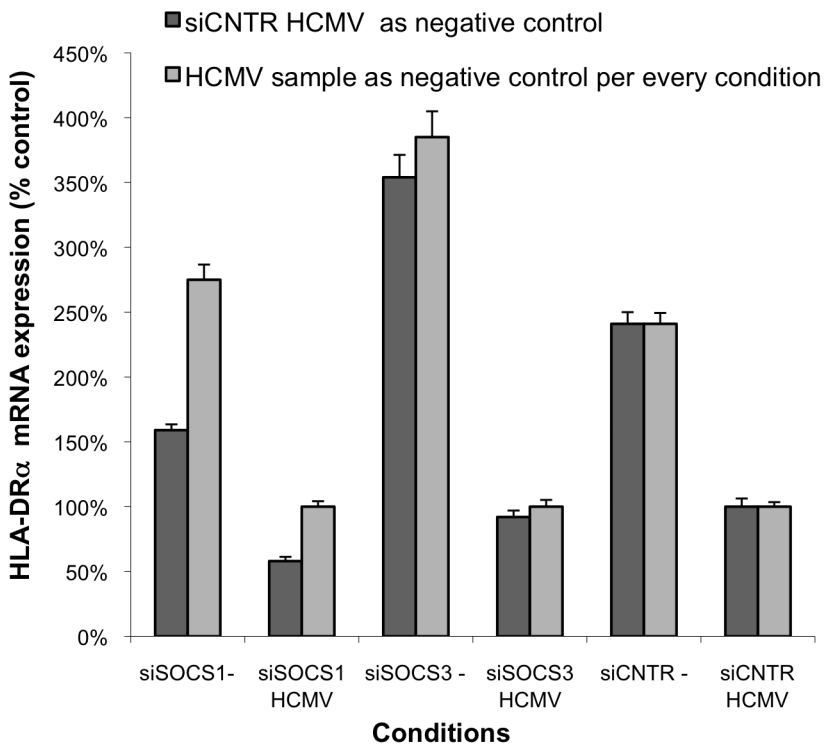

Figure 5.5: Reduction of IFNγ-induced HLA-DRα mRNA expression upon HCMV infection is not dependent on SOCS1 or SOCS3

HMVEC transfected with siSOCS1, siSOCS3 or siCNTR were infected with HCMV at an MOI of 5 (samples labeled 'HCMV'). Non-infected or 2 days p.i. cultures were stimulated for 24 hours with 100U/ml IFNγ. Transcription of HLA-DRα mRNA was reduced in all samples upon infection (light gray columns, infected cultures within the conditions as reference). The reduction of HLA-DRα expression reached the same level in all the samples (dark gray columns, related to siCNTR HCMV). Shown is the mean with SD of one representative experiment carried out in duplicate as percentage to the respective control.

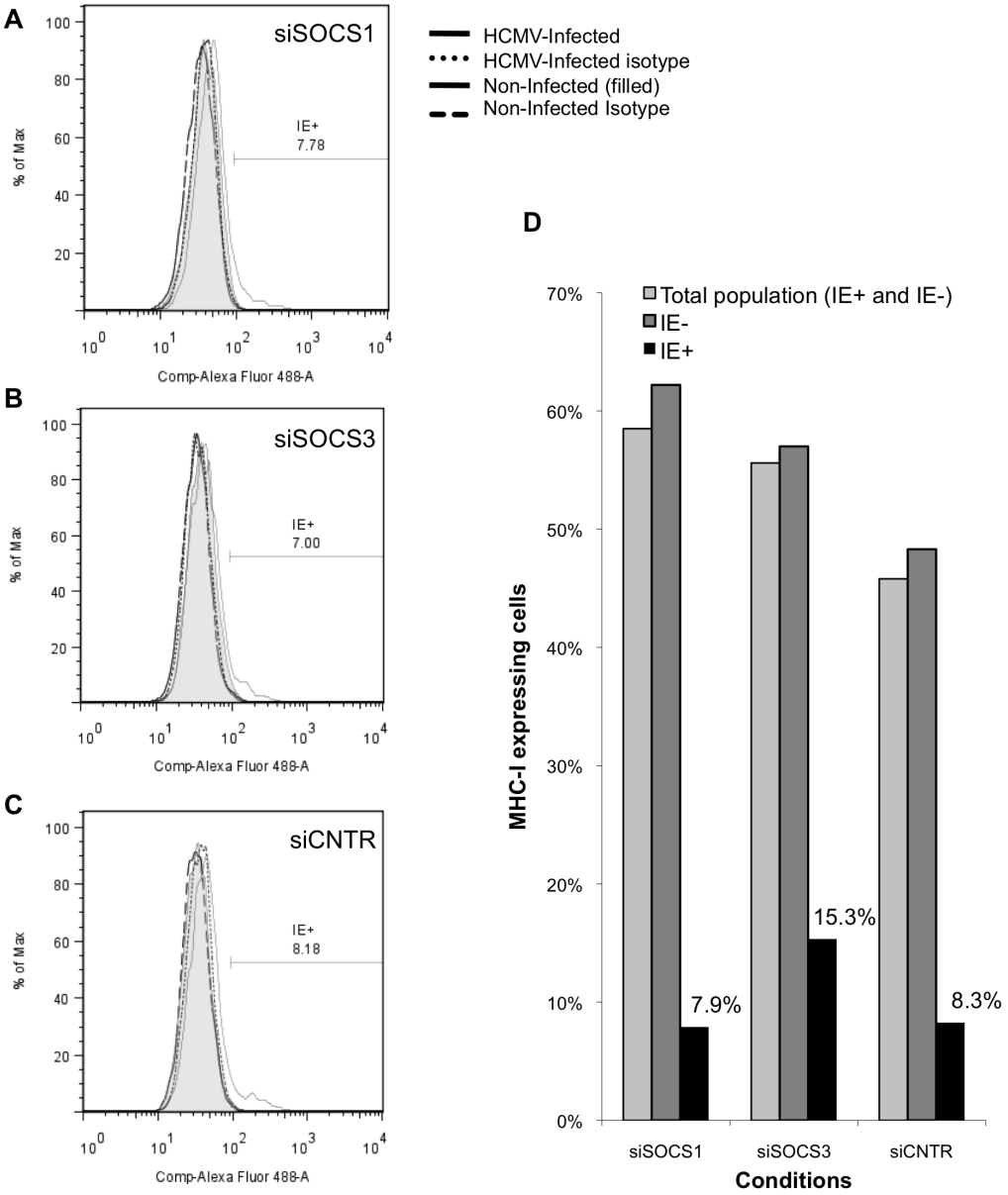

Figure 5.6 Reduction of IFNβ-induced MHC-I expression upon HCMV infection is influenced by silencing of SOCS3

HMVEC transfected with siSOCS1, siSOCS3 or siCNTR were infected with HCMV for 48 hours and then stimulated with 500U/ml IFNβ fro 24 hours. Analyses of the samples by FACS revealed that 3 days p.i. the number of cells infected (IE+) was the same for siSOCS1 (A) siSOCS3 (B) and siCNTR (C) cultures. Expression of MHC-I within the infected fraction (black) revealed that in siSOCS3 cultures the percentage of cells expressing MHC-I is increased in comparison to the two remaining conditions (D). MHC-I expression for the total population or IE- cells are shown in light and middle gray and demonstrated an expected upregulation in all three conditions.

5.5 Discussion

In this study we demonstrated that HCMV needs SOCS3 for an efficient replication in HMVEC. We started by investigating the ability of these cells to express and modulate the expression of SOCS1 and SOCS3. Stimulation of cells with PMA or IFNγ resulted in mRNA increase for both SOCS1 and SOCS3 whereas only SOCS3 was found to be upregulated on the protein level (Figure 5.1). This apparent discrepancy between the mRNA and the protein expression of SOCS1 was also found in HCMV infected cells where the virus was able to considerably upregulate SOCS1 mRNA between 8 and 12 hours p.i. but no modulation of the protein was observed (Figure 5.2).

A similar phenomenon was recently reported for vesicular stomatitis virus (VSV). Cells infected with VSV showed an upregulation of SOCS1 mRNA but not for the protein [102]. This result correlated with a negative posttranscriptional regulation of SOCS1 translation by microRNA-155 (miR-155). Emerging relevance of miR-155 as regulator of SOCS1 has been recently published [98-103]. Expression of miR-155 is induced by toll like receptor (TLR) signals (TLR2, 3, 4 and 9) and by stimulation (IL-1, TNFα, PMA, IFNβ and γ) indicating that this SOCS1 modulator is expressed in the context of inflammation [104-108]. Moreover EBV and Kaposi's sarcoma-associated herpes virus (KSHV), two member of the herpesvirus family, have already been found to induce expression of miR-155 [180] or to directly express an ortholog to miR-155 [181], respectively. We are currently investigating the potential role of miR-155 in our model.

SOCS3 mRNA transcription was not modulated by HCMV but an accumulation of the protein was observed during infection (Figure 5.2). This accumulation was found to be dependent on active replication of the virus since it was not observed in UV-inactivated HCMV infected culture, where HCMV can enter the cell but cannot replicate. Modulation of the half-life of SOCS3 has been shown to be dependent on the binding of ubiquitin machinery to the SOCS box with considerable extension of the half-life of the protein through stabilization of the complex [110,123,124]. Moreover SOCS3 is the only SOCS protein containing a 35 amino acid PEST [125] (Proline (P), Glutamate (E), Aspartate (D), Serine (S) and Threonine (T)) motive which has been described to be recognized as a site for protein degradation [126] and for SOCS3 it has been found to be responsible for the cellular turnover of this protein. In fact, removing PEST drastically increased the half-life of SOCS3 without

compromising its activity [125]. The exact mechanism of the observed accumulation in our model remains open and may involve inhibition of protein degradation. We suggest that SOCS3 may be stabilized by the infection through binding, by viral or viral modulated cellular factors, of the key regions (i.e. PEST, SOCS box-ubiquitin machinery complex) involved in turn over regulation of the protein resulting in an elongated half-life and therefore in an accumulation of SOCS3 in infected cells.

Importance of SOCS1 and SOCS3 for HCMV replication in HMVEC was assessed by silencing experiments. Upon silencing of SOCS3, HCMV showed a limited replication capacity in HMVEC. Inhibition of IE, E and L viral antigen production was observed showing an especially high decrease of L antigen and a partial reduction of E antigen whereas IE antigen production was not much impacted (Figure 5.3). In accordance, and such demonstrating a profound impact on viable virus, the HCMV titer collected in siSOCS3 cultures were significantly reduced in comparison with siSOCS1 and siCNTR cultures. These results suggest a possible role of SOCS3 relatively late in the infection. In line with this, cells transfected just after infection also showed a similar reduction of both viral antigens and infectious viral particles produced (data not shown). Moreover the ratio of infected cells detected 1 day p.i. (data not show) and 3 days p.i (Figure 5.6A, B and C) based on IE staining was the same in all the three conditions (siSOCS1, siSOCS3 and siCNTR). These results indicate that SOCS3 plays a role only later after infection, since the virus can enter and infect the same number of cells in all three conditions.

Analysis of the activation pattern of infected cultures revealed an increase in STAT1 phosphorylation for siSOCS1 and siSOCS3 cultures compared to siCNTR (Figure 5.4), which was stronger 2 days p.i. than 5 days p.i. and did not depend on the MOI of the inoculum. These results indicate a possible role for SOCS3 or SOCS1 in type II IFN escape mechanism of the virus since STAT1 is a major component of this pathway. Therefore we investigated the ability of HCMV to lower the transcription of HLA-DRα, an MHC-II gene, upon IFNγ stimulation as shown previously [36,38,39]. The results showed a similar ability of the virus to downregulate the expression of HLA-DRα in all the three condition (Figure 5.5), indicating that control of at least MHC-II gene expression occurs independently from SOCS1 or SOCS3.

Phosphorylation of STAT2 on the contrary was found to be specific for siSOCS3 infected cultures and this increase was observed only in cells that were infected with

an MOI of 5 indicating a probable relation between STAT2 phosphorylation and number of infected cells (Figure 5.4).

These results suggested an involvement of SOCS3 in the inhibition of STAT2 phosphorylation by HCMV. Since STAT2 is a major component with STAT1 of the type I IFN pathway we decided to evaluate the ability of the virus to control this pathway in siSOCS3 cells. To evaluate the impact of STAT2 rescued phosphorylation and HCMV ability to control type I IFN pathway in siSOCS3 infected cells, we stimulated cells with IFNβ and measured expression of MHC-I molecules known to be down regulated in HCMV infected cells [42]. The fraction of infected siSOCS3 cells expressing MHC-I molecules was about two times higher compared with siSOCS1 or siCNTR (Figure 5.6D). From previous experiments it was known that HCMV could inhibit the phosphorylation of STAT2 in TB40/E infected fibroblast. In cultures infected at a very high rate even degradation of STAT2 was observed. Following restoration of STAT2 expression, phosphorylation and activation by type I IFN was still impaired indicating two different layers of HCMV interference with STAT2 activation, one controlling the phosphorylation and one degrading the protein [50]. Based on our results we suggest that SOCS3 is an important component for the HCMV-mediated control of STAT2 phosphorylation. In other studies carried out with the Towne strain on fibroblast it has been shown that IE72 interact with STAT2 [51,182] and implication in the control of STAT2 by IE has been provided. HCMV has develop many strategies to control IFN activation [9]. Depending on the cell type and on the virus strain these mechanisms vary resulting in a complex multistep process. In our model we demonstrate that reduction of SOCS3 not only leads to restoration of STAT2 phosphorylation but also increases the response of infected cells to type I IFN. These results indicate, for the first time, an involvement of SOCS3 in HCMV mediated inhibition of type I IFN pathway and may also suggest a possible role of SOCS3 in the process of latency since MHC-I molecules downregulation is essential to escape $CD8^+$ T-Cells mediated clearance.

In summary we describe here for the first time that SOCS3 plays an essential role during HCMV replication in primary human endothelial cells. Accumulation of SOCS3 protein was observed in infected cells and a correlation between SOCS3 and the ability of the virus to efficiently replicate was provided. Indications of a SOCS3-dependend mechanism of HCMV to control type I IFN activation through inhibition of STAT2 phosphorylation were given.

These findings add a novel aspect to the biology of HCMV and potentially identify SOCS3 as novel target protein to control of HCMV viral replication.

5.6 Materials and methods

5.6.1 Cells and viruses

All experiments were carried out in primary human lung-derived microvascular endothelial cells (HMVEC, Lonza, Basel, Switzerland) between passage 4 and 8, cultured in complete EGM-2 medium (Lonza, Basel, Switzerland).

All experiment were carried out with the HCMV strain TB40/E [183], kindly provided by Dr. Christian Sinzger (University of Ulm, Germany). The virus was propagated on human lung-derived fibroblasts MRC5 [184] (LGC Standards, Teddington, UK, cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Basel, Switzerland) containing 10% fetal calf serum (A15-101, lot: A10110-2432, PAA) and 2mM L-glutamine (Invitrogen). Purification of HCMV was carried out by ultracentrifugation over a 15% sucrose cushion (50mM Tris-HCl, 12mM KCl, 5mM Na_2EDTA) at 20,000g for 90 min at 4°C using an SS-34 rotor (Beckman Coulter, Fullerton, CA). The infectious titer in HCMV for the stock virus and for supernatant of the experiments was determined by TCID50 assays on MRC5 [185]. As control, HCMV was UV-inactivated (5 min, 30 cm distance from a 30W, 230V, 50Hz UV lamp (Osram, Winterthur, Switzerland)) [186].

HMVEC were infected with either a MOI of 1 or MOI of 5. If not else indicated the infection rate was determined one day p.i. by immunofluorescence as previously described [26] employing an antibody against immediate early 52, 72 and 86 kDa proteins (Ref. 11-003, Argene, Varhiles, France), resulting in about 7-12% of infected cells for MOI of 1 and 30-40% for MOI of 5 in all the experiments showed. All cell preparations were tested negative by 4, 6-diamidino-2-phenylindole (DAPI; D9542, Sigma, Buchs, Switzerland) staining for mycoplasma.

Cells were stimulated for the period of time indicated for each experiment with 500ng/ml PMA (Sigma), 100U/ml IFNγ (PeproTech, Rocky Hill, NJ) or 500U/ml IFNβ (PeproTech). HMVEC were infected one day post transfection in all the silencing experiments.

5.6.2 Real-Time PCR

Total RNA from cells was isolated using RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) with DNase (RNase-Free DNase Set, Qiagen) treatment step. Superscript III reverse transcriptase (Cat. No. 18080-093, Invitrogen) was used, following manufacturer's instruction, for reverse transcription of the mRNA. cDNA was analyzed with Taqman gene expression assays (Applied Biosystem) for SOCS1 (Assay ID: Hs00705164_s1), SOCS3 (Assay ID: Hs00269575_s1), HLA-Drα (Assay ID: Hs00219575_m1) and GAPDH (Assay ID: Hs9999905_m1) using HotStarTaq master mix (Qiagen) supplemented $MgCl_2$ (1.5 μM). Amplification of cDNA was performed with a real time thermocycler (iQ5 Cycler; Bio-Rad, Reinach, Switzerland) as follows: 15 min at 95 °C and 45 cycles of 15 second at 95°C and 60 seconds at 60°C. GAPDH was used as housekeeping genes [187] to normalize results, data were analyzed using the iQ5 Optical System Software (Bio-Rad), and results were reported as relative expression levels to the untreated controls.

5.6.3 Western blot

Western blot analysis of SOCS1 (#3950, working dilution 1/300, Cell Signaling), SOCS3 (sc-51699, working dilution 1/500, Santa Cruz Biotechnology, Heidelberg, Germany), STAT1P (Tyr701) (sc-136229, working dilution 1/500, Santa Cruz Biotechnology) STAT1 (sc-346, working dilution 1/500, Santa Cruz Biotechnology) , STAT2P (Tyr690) (#4441, working dilution 1/500, Cell Signaling,), β-Actin (A5441, working dilution 1/5000, Sigma) and of the IE (52, 72 and 86kDa proteins, Ref 11-003, working dilution 1/5000, Argene), E (ICP36, CA006-100, working dilution 1/10000, Virusys, Taneytown, MD), L (pp65, vp-c 422, working dilution 1/1000, Vector Laboratories, Burlingame, CA) viral antigen were carried out as follow. Cell lysates were prepared by adding 50 μl of ice-cold lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 1% Tween 20, 1mM EDTA, 0.1% SDS, and protease inhibitor, Calbiochem) to the monolayer and collected allowing lysis for 30 minutes. Protein concentrations were determined by Nanodrop 1000 spectrophotometer. Everything was carried out strictly on ice until SDS sample buffer (50mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.1% Bromophenol dye) was added to the samples which were then boiled at 95°C for 5 minutes. Equal amounts of

protein from cell lysates were separated by gel electrophoresis and transferred to a polyvinyl membrane (GE Healthcare, Muenchen, Germany). After blocking with 5% milk powder (Rapilait, Migros, Switzerland) in 1x Tween 20/Tris-buffered saline (0.1% Tween 20, 25 mM Tris–HCl, pH 7.5, and 150 mM NaCl) for 2 h at room temperature, membranes were incubated with the primary antibody overnight at 4 °C, followed by anti mouse or anti rabbit horseradish peroxidase-conjugated secondary antibody (working dilution 1:5000) (GE Healthcare) for 1 h. The target proteins were detected by enhanced chemiluminescence using the ECL reagent (GE Healthcare). Signals were quantified with the ImageJ analysis software.

5.6.4 Cells transfection

Small interfering RNA (siRNA) targeting SOCS1 mRNA (HSS189479, stealth RNAi, Invitrogen use in the experiment shown and sc-40997, Santa Cruz Biotechnology to confirm the results) or SOCS3 mRNA (HSS113313, stealth RNAi, Invitrogen experiment shown and sc-41000, Santa Cruz Biotechnology to confirm the results) and a scramble non-specific sequence (CNTR-D or A sc-44232 and sc-37007 respectively, Santa Cruz Biotechnology) were transfected using Lipofectamin RNAiMax (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in a 24 well plate. 50 ρmol of siRNA were mixed with 1.5 μl Lipofectamin RNAiMax in 100 μl OptiMEM (Invitrogen), incubated for 10 minutes at room temperature before adding the mixture to the cells.

5.6.5 Cell staining and flow cytometry

Cells were harvested with a mild trypsin (Invitrogen, diluted to final concentration of 0.005% in 0.02%EDTA) treatment. For combined cell surface and IE stainings, cells were first stained with PE conjugated mAb to HLA-ABC (G46-2.6, BD Pharmingen, San Diego, CA). Next, cells were fixed and permeabilized following the BD Cytofix/Cytoperm^{TM} protocol (Becton-Dickinson), and then incubated with Alexa-fluor 488 conjugated IE1 mAb (8B1.2, Merck Millipore, Darmstadt, Germany). The PE conjugated G155-178 (IgG2ak, BD Pharmingen) and Alexa-fluor conjugated normal mouse IgG (IgG, Merck Millipore) were used as isotype-matched controls. The fluorescence intensity was measured by using a FACSCanto (BD Biosciences, Basel, Switzerland) and analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

5.6.6 Statistical analysis

Significances of TCID50 values (Figure 3D) were calculated with the Fisher's exact test performed with StatView software (SAS institute Inc., San Francisco, CA) with $p<0.05$ (*) and $p<0.0001$ (**).

5.6.7 Author contributions

OS and NJM conceived the basic concept and designed the experiments. OS and AMT performed the experiments and analyzed the data. ALM, LD, RFS and NJM contributed to the analysis of data and development of new ideas. OS and NJM wrote the paper. All authors critically revised it.

5.6.8 Funding

This work was supported by a research grant from the Swiss National Science Foundation to NM (310030_130705).

5.6.9 Competing interests

The authors have declared no competing interests.

5.6.10 Acknowledgment

We would like to thank Dr. Christian Sinzger (University of Ulm, Germany) for kindly providing us the HCMV TB40/E strain.

6. General discussion

6.1 Porcine setting

In the last decades major findings have led to an increased understanding of molecular mechanism underlying immunological processes. Together with the discovery of potent immunosuppressive compounds, transplantation has become a clinical reality. This success has created a new problem: the shortage of organs. Alternatives are intensely investigated. Transplantation of pig organs into humans has been advocated as a potential solution [188]. Clinical trial are already performed using porcine islet cells for diabetic patients [189]. In anticipation of a clinical application issues such as compatibility [190] and zoonosis [191] are major hurdles. This laboratory established [26] and characterized [27] a model of HCMV infection of porcine cells to evaluate the possibility and the impact of a cross species infection. Based on the work of Maddalena Ghielmetti [169] who established a RT-PCR system for the detection of porcine SOCS1 and SOCS3 we evaluated the modulation of these two transcripts in porcine cells upon HCMV infection. Interestingly, only primary cells (PAEC-KO) were found to upregulate SOCS1 mRNA transcription as from 24 hours p.i. on. The other cell lines tested did not reveal any particular modulation of SOCS1, whereas SOCS3 was not modulated in any of the cell types evaluated. Considering the role of endothelial cells at the interface between recipient blood and grafted tissue, exposed to numerous stimuli, it is reasonable to postulate that these cells need to be able to quickly and efficiently modulate responses to danger signals to ensure homeostasis.

It also revealed the importance of the model, i.e. cell, chosen. The process of immortalization results in profound changes in the metabolism of the cell [192]. Expression of molecules involved in cell proliferation, regulation of homeostasis and cell cycle are changed [193,194]. Given that SOCS1 and SOCS3 are involved in a number of processes controlling the response to stimuli, especially via JAK/STAT, it is likely that changes in the expression pattern of these proteins may be related to the process of immortalization. Supporting this idea is the fact that in human cancer expression and modulation of SOCS1 and SOCS3 is altered [81,195]. We used HeLa cells [196] as positive control for SOCS1 and SOCS3 expression, one of the

oldest cell line generated from a cervix cancer and with considerable expression of both SOCS1 and SOCS3.

All these findings implied that using immortalized cell lines to study the involvement of SOCS1 and SOCS3 in HCMV infection was prone to a number of potential problems. Supporting this concept results collected with the cell line MRC5 did not show any remarkable modulation of SOCS1 or SOCS3 upon HCMV infection.

The upregulation of SOCS1 mRNA found in HCMV PAEC KO prompted us to investigate the protein level for both SOCS1 and SOCS3. Since none of the commercially-available antibodies were targeting porcine SOCS1 or SOCS3, we screened selected antibodies designed against human SOCS based on the higher than 90% homology between the porcine and the human SOCS1 and SOCS3. Two antibodies did recognize a band of the size of SOCS1, or SOCS3, respectively. Obtaining consistent results was very difficult, and probably due to the fact that cross-reactivity was limited. Encouraged by the intriguing finding of SOCS modulation in pEC, we shifted our investigation to the possible role of SOCS1 and SOCS3 during HCMV infection in human endothelial cells.

6.2 Human setting

6.2.1 Detection and induction of SOCS

A number of viruses have been shown to exploit SOCS proteins to their advantage, among them EBV and the HSV-1, two members of the herpesvirus family. As initial investigation, a system for detection for SOCS1 and SOCS3 mRNA by the RT-PCR was developed. MRC5 and HMVEC were stimulated with known inducers IFNγ, PMA, or left untreated to evaluate the constitutive transcription rate and the possibility to modulate the mRNA of SOCS1 and SOCS3 expression. All cells tested expressed a basal level of SOCS1 and SOCS3 with modulation of the transcription with different stimuli. Response to the different stimuli was cell type dependent. Stimulation of MRC5 with PMA resulted in a strong upregulation of SOCS3 mRNA (about 60-fold as compared to mock control) and in a modest 10-fold induction of SOCS1. PMA-stimulated HMVEC showed an opposite trend where SOCS1 mRNA was highly (about 100-fold) expressed and SOCS3 modestly (about 15-fold)

modulated. A complete different pattern of expression was observed when MRC5 or HMVEC were stimulated with IFNγ. These results were a first sign hinting at the complexity of SOCSs interactions in different cells. This was confirmed by the fact that upon HCMV infection the rates of SOCS1 and SOCS3 expression were different in time and magnitude for the different cell type tested.

Analysis of SOCS1 and SOCS3 proteins by western blot yielded an unexpected result. Despite the upregulation of the mRNA level for SOCS1 after HCMV infection, the protein level was not modulated or even decreased. This discrepancy was recently described by other groups who had observed this phenomenon for SOCS1. A possible link was found in the expression of a microRNA called mIR-155 [102]. mIR-155 was first identified as a putative miRNA targeting SOCS1. Based on the sequence and after further characterization a clear role for mIR-155 in targeting and inhibiting SOCS1 expression was documented [98-103]. In cells infected with VSV, SOCS1 mRNA expression was about 20 times higher than in non-infected cells but the protein level was reduced. This was correlated with the fact that infection of VSV upregulated mIR-155 expression which inhibits the translation of SOCS1 [102]. mIRNA-155 is induced by a number of different stimuli in the context of inflammation [104-108], and has been found to be expressed upon EBV infection [180] and KSHV, which carries in its genome a homologue of mIR-155 [181]. We hypothesize that the phenomenon we are observing in HMVEC may be due to the result of a post translational regulation driven by mIRNA-155. It will be of great interest to further investigate this point to test this hypothesis and elucidate the mechanism behind this apparent discrepancy.

On the contrary, SOCS3 mRNA showed a correlation between mRNA upregulation observed upon stimulation and level of protein detected. Particularly intriguing is the fact that upon infection with HCMV, on the contrary, we did not detect any SOCS3 mRNA modulation despite an increase in SOCS3 protein observed. SOCS3 and the SOCS protein in general are believed to have a fast cellular turnover. The stability of SOCS3 and thus its half-life can be improved and extended in several manners. Under stress conditions SOCS3 mRNA can be translated from different starting points increasing the translation level of the protein without compromising its activity. These alternative start codon lies just after a SOCS3 ubiquitination site, thus starting the translation from this codon generates a fully functional SOCS3 protein lacking an ubiquitination site resulting in a longer half-life [122]. Infection constitutes such a

stress and the alternative translation start points could be an explanation for the increased quantity of SOCS3 during infection in HMVEC. Stability of SOCS3 it is also mediated by the SOCS box. Modulation of the half-life of SOCS3 has been shown to be dependent on the binding of the ubiquitin machinery to the SOCS box with considerable extension of the half-life of the protein through stabilization of the complex [110,123,124]. Moreover, SOCS3 is the only SOCS protein containing a 35 amino acid PEST [125] motive which has been described to be recognized as a site for protein degradation [126]. For SOCS3, this motive has been shown to be responsible for the cellular turnover of the protein. Removing PEST drastically increased the half-life of SOCS3 without compromising its activity [125]. It is possible that specific viral proteins can either stabilize the binding between SOCS box and ubiquitin machinery and/or bind to the PEST region avoiding tagging of SOCS3 for degradation. How HCMV infection causes this increase of SOCS3 protein remains unknown.

6.2.2 Silencing of SOCS1 and SOCS3

To further investigate the role of SOCS1 and SOCS3 in HCMV-infected HMVEC, silencing of the two genes was attempted to investigate the impact on the viral replication. Initially, western blot analysis of the proteins was not pointing to a successful silencing. Nevertheless, silenced cultures were infected with HCMV and viral antigen and infectious particles productions assessed. In both siSOCS1 and siSOCS3 conditions, a decrease in both viral antigen and progeny productions was observed. Similar results were seen in cultures silenced twice (two day pre infection and 4 days p.i.) indicating that the first silencing was sufficient. Knowing that silencing of primary cells can pose a challenge due to their refractory nature to transfection and their fragility, we needed to repeat these experiments and ensure that the effect observed was specific for the silenced genes and not an unspecific result of application of siRNA. With these experiments a first read out was provided, showing an impact on HCMV replication upon downregulation of SOCS1 and SOCS3. The quality of the western blot however did not allow determining the magnitude of the downregulation of the target proteins. In addition, based on these blots, a possible cross-reactivity between siRNA targeting SOCS3 and SOCS1 could not be excluded. Optimization of the western blot technique was crucial. Quantification of the western blot samples via nanodrop allowed loading the same

quantity of cells lysate for every condition and every time point. Extraction of the proteins on ice with a specific LB improved the signal of the target proteins allowing defining a threshold necessary to detect SOCS1 and SOCS3. Since SOCS are negative regulators of JAK/STAT pathways a control to detect possible preactivation of the type I IFN pathway via detection of Mx protein was introduced. Mx protein is one of the classical type I IFN induced proteins. The optimization revealed two problems: the first was that siRNA against SOCS3 also was downregulating SOCS1 to a lesser extent, and the second was that the silencing settings used to produce to first results with the virus did induce Mx production. Activation of IFN type I can indeed produce misleading resultes with the possibility that the reduction in viral replication was due to the activation of type I IFN and not SOCS1 or SOCS3 downregulation. An alternative explanation could be that the downregulation of the target proteins by themselves lead to an activation of the type I IFN pathway since mock silencing revealed inconstant amount of Mx proteins. Another important role was played by the age of the cells. Primary cells can duplicate only a certain number of times before senescence and we noticed that employing cells older than passage 8 for silencing experiment further increased Mx induction. Solving this issue was mandatory as well as optimizing the specificity of the silencing. To achieve these purposes different strategies were evaluated. Protocols of transfection were varied, from the forward one we usually employed to the reverse transfection believed to be less invasive. Evaluation of other alternative transfection regencies was attempted. Unspecific induction of antiviral states as measured by Mx protein was avoided, but silencing was poor. To overcome the difficulties a novel approach for the downregulation of the target proteins was evaluated, employing a lentiviral transduction system. This system is based on lentiviral delivery of DNA. The system is based on modified lentiviral particles carrying a plasmid that encodes for shRNA targeting a chosen gene [197]. This system has many advantages compared to the conventional lipofection approach. It is based on shRNA which are more stable than siRNA introduced into cells by transfection, and does not need any transfection reagent which is much less invasive and toxic. shRNA coding DNA is integrated into the cellular genome and potentially allows downregulation of the target protein for an unlimited time. The plasmid carrying the shRNA coding DNA is linked to a GFP coding box allowing to determine the rate of transduced cells. The transduction efficiency was assessed with the wild type vector, since no previous study had

shown that HMVEC were transducible with these plasmids combinations. The first results were encouraging since a very high rate of GFP positive cells (about 90%) was seen. Two shRNA specific for SOCS1, one for SOCS3 and a scramble control were designed. After cloning of the sequences into the delivery vectors and packaging of the plasmids into the pseudoviral particles, HMVEC were transduced with the different construct. Analysis of the samples by western blot revealed that despite a high rate of transduced cells (about 80-90%) no downregulation of the target proteins was seen. It is known that certain sequences work for a given type of cell but fail in others.

The breakthrough came when the initial classic method with lipofection and siRNA was used again with siRNA from a different company. A specific silencing for SOCS1 and SOCS3, and with no type I IFN activation of the cells was finally achieved. The experiments were all repeated with the new setting. We were able to confirm that silencing of SOCS3 reduces HCMV replication efficiency in HMVEC and, in contrast with what we observed with the old silencing setting, SOCS1 downregulation did not impact the replication of HCMV in HMVEC. The previous observation was most probably due to the type I IFN activation of the cells.

The main results are summarized and discussed in the Chapter 5.

6.3 Conclusion

These results for the first time reveal a role for SOCS proteins in HCMV infection. We have only started to elucidate the potential mechanisms behind these findings. It will be of interests to understand the mechanism behind HCMV-SOCS3 protein interference in infected cells and to investigate the role of SOCS3 *in* or *ex vivo*. These results will not only expand our knowledge on the biology of HCMV but may be a first step to develop novel therapeutical strategies targeting SOCS3.

7. References

1. Landolfo S, Gariglio M, Gribaudo G, Lembo D (2003) The human cytomegalovirus. Pharmacology & Therapeutics 98: 269–297. doi:10.1016/S0163-7258(03)00034-2.

2. Coen DM, Schaffer PA (2003) Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets. Nat Rev Drug Discov 2: 278–288. doi:10.1038/nrd1065.

3. Hakki M, Chou S (2011) The biology of cytomegalovirus drug resistance. Current Opinion in Infectious Diseases 24: 605–611. doi:10.1097/QCO.0b013e32834cfb58.

4. Schreiber A, Härter G, Schubert A, Bunjes D, Mertens T, et al. (2009) Antiviral treatment of cytomegalovirus infection and resistant strains. Expert Opin Pharmacother 10: 191–209. doi:10.1517/14656560802678138.

5. Härter G, Michel D (2012) Antiviral treatment of cytomegalovirus infection: an update. Expert Opin Pharmacother 13: 623–627. doi:10.1517/14656566.2012.658775.

6. Isaacson MK, Juckem LK, Compton T (2008) Virus entry and innate immune activation. Curr Top Microbiol Immunol 325: 85–100.

7. Soroceanu L, Akhavan A, Cobbs CS (2008) Platelet-derived growth factor-α receptor activation is required for human cytomegalovirus infection. Nature 455: 391–395. doi:10.1038/nature07209.

8. Compton T, Mary HT (2002) Human cytomegalovirus glycoprotein O as a new drug target and subunit vaccine candidate.

9. Marshall EE, Geballe AP (2009) Multifaceted Evasion of the Interferon Response by Cytomegalovirus. Journal of Interferon & Cytokine Research 29: 609–620. doi:10.1089/jir.2009.0064.

10. Arvin A, Campadelli-Fiume G, Mocarski E, Moore PS, Roizman B, et al. (2007) Betaherpes viral genes and their functions. Cambridge: Cambridge University Press. pp.

11. Tomtishen JP (2012) Human cytomegalovirus tegument proteins (pp65, pp71, pp150, pp28). Virol J 9: 22. doi:10.1186/1743-422X-9-22.

12. Mettenleiter TC (2002) Herpesvirus assembly and egress. Journal of Virology 76: 1537–1547.

13. Sinzger C, Kahl M, Laib K, Klingel K, Rieger P, et al. (2000) Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus. J Gen Virol 81: 3021–3035.

14. Streblow DN, Orloff SL, Nelson JA (2007) Acceleration of allograft failure by cytomegalovirus. Curr Opin Immunol 19: 577–582. doi:10.1016/j.coi.2007.07.012.

15. Pérez-Sola MJ, Castón JJ, Solana R, Rivero A, Torre-Cisneros J (2008) Indirect effects of cytomegalovirus infection in solid organ transplant recipients. Enferm Infecc Microbiol Clin 26: 38–47.

16. Snydman DR, Limaye AP, Potena L, Zamora MR (2011) Update and review: state-of-the-art management of cytomegalovirus infection and disease following thoracic organ transplantation. Transplant Proc 43: S1–S17. doi:10.1016/j.transproceed.2011.02.069.

17. Lemonovich TL, Watkins RR (2012) Update on cytomegalovirus infections of the gastrointestinal system in solid organ transplant recipients. Curr Infect Dis Rep 14: 33–40. doi:10.1007/s11908-011-0224-6.

18. Loebe M, Schüler S, Zais O, Warnecke H, Fleck E, et al. (1990) Role of cytomegalovirus infection in the development of coronary artery disease in the transplanted heart. J Heart Transplant 9: 707–711.

19. Westall GP, Michaelides A, Williams TJ, Snell GI, Kotsimbos TC (2003) Bronchiolitis obliterans syndrome and early human cytomegalovirus DNAaemia dynamics after lung transplantation. Transplantation 75: 2064–2068. doi:10.1097/01.TP.0000069234.04901.A3.

20. Madalosso C, de Souza NF, Ilstrup DM, Wiesner RH, Krom RA (1998) Cytomegalovirus and its association with hepatic artery thrombosis after liver transplantation. Transplantation 66: 294–297.

21. Nett PC, Heisey DM, Fernandez LA, Sollinger HW, Pirsch JD (2004) Association of cytomegalovirus disease and acute rejection with graft loss in kidney transplantation. Transplantation 78: 1036–1041.

22. Varga M, Remport A, Czebe K, Péter A, Toronyi E, et al. (2008) [Cytomegalovirus infection after solid-organ transplantation, its risk factors, direct and indirect effects and prevention strategies]. Orv Hetil 149: 551–558. doi:10.1556/OH.2008.28324.

23. Castón Osorio JJ, Zurbano Goñi F (2011) [Indirect effects of cytomegalovirus infection]. Enferm Infecc Microbiol Clin 29 Suppl 6: 6–10. doi:10.1016/S0213-005X(11)70050-7.

24. Mueller NJ, Barth RN, Yamamoto S, Kitamura H, Patience C, et al. (2002) Activation of cytomegalovirus in pig-to-primate organ xenotransplantation. Journal of Virology 76: 4734–4740.

25. Degré M, Ranneberg-Nilsen T, Beck S, Rollag H, Fiane AE (2001) Human cytomegalovirus productively infects porcine endothelial cells in vitro. Transplantation 72: 1334–1337.

26. Millard A-L, Häberli L, Sinzger C, Ghielmetti M, Schneider MKJ, et al. (2010) Efficiency of porcine endothelial cell infection with human cytomegalovirus depends on both virus tropism and endothelial cell vascular origin. Xenotransplantation 17: 274–287. doi:10.1111/j.1399-3089.2010.00594.x.

27. Ghielmetti M, Millard A-L, Haeberli L, Bossart W, Seebach JD, et al. (2009) Human CMV Infection of Porcine Endothelial Cells Increases Adhesion Receptor Expression and Human Leukocyte Recruitment. Transplantation 87: 1792–1800. doi:10.1097/TP.0b013e3181a75a41.

28. Sinzger C, Digel M, Jahn G (2008) Cytomegalovirus cell tropism. Curr Top Microbiol Immunol 325: 63–83.

29. Fish KN, Stenglein SG, Ibanez C, Nelson JA (1995) Cytomegalovirus persistence in macrophages and endothelial cells. Scand J Infect Dis Suppl 99: 34–40.

30. Jarvis MA, Nelson JA (2002) Human cytomegalovirus persistence and latency in endothelial cells and macrophages. Curr Opin Microbiol 5: 403–407.

31. Myerson D, Hackman RC, Nelson JA, Ward DC, McDougall JK (1984) Widespread presence of histologically occult cytomegalovirus. Hum Pathol 15: 430–439.

32. Sinzger C, Grefte A, Plachter B, Gouw AS, The TH, et al. (1995) Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J Gen Virol 76 (Pt 4): 741–750.

33. Wiley CA, Schrier RD, Denaro FJ, Nelson JA, Lampert PW, et al. (1986) Localization of cytomegalovirus proteins and genome during fulminant central nervous system infection in an AIDS patient. J Neuropathol Exp Neurol 45: 127–139.

34. Fish KN, Soderberg-Naucler C, Mills LK, Stenglein S, Nelson JA (1998) Human cytomegalovirus persistently infects aortic endothelial cells. Journal of Virology 72: 5661–5668.

35. Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, et al. (2006) Human Cytomegalovirus (HCMV) Infection of Endothelial Cells Promotes Naive Monocyte Extravasation and Transfer of Productive Virus To Enhance Hematogenous Dissemination of HCMV. Journal of Virology 80: 11539–11555. doi:10.1128/JVI.01016-06.

36. Le Roy E, Mühlethaler-Mottet A, Davrinche C, Mach B, Davignon JL (1999) Escape of human cytomegalovirus from HLA-DR-restricted CD4(+) T-cell response is mediated by repression of gamma interferon-induced class II transactivator expression. Journal of Virology 73: 6582–6589.

37. Baron M, Davignon J-L (2008) Inhibition of IFN-gamma-induced STAT1 tyrosine phosphorylation by human CMV is mediated by SHP2. The Journal of Immunology 181: 5530–5536.

38. Miller DM, Rahill BM, Boss JM, Lairmore MD, Durbin JE, et al. (1998) Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. J Exp Med 187: 675–683.

39. Sedmak DD, Guglielmo AM, Knight DA, Birmingham DJ, Huang EH, et al. (1994) Cytomegalovirus inhibits major histocompatibility class II expression on infected endothelial cells. Am J Pathol 144: 683–692.

40. Knight DA, Waldman WJ, Sedmak DD (1997) Human cytomegalovirus does not induce human leukocyte antigen class II expression on arterial endothelial cells. Transplantation 63: 1366–1369.

41. Scholz M, Hamann A, Blaheta RA, Auth MK, Encke A, et al. (1992) Cytomegalovirus- and interferon-related effects on human endothelial cells. Cytomegalovirus infection reduces upregulation of HLA class II antigen expression after treatment with interferon-gamma. Hum Immunol 35: 230–238.

42. Miller DM, Zhang Y, Rahill BM, Waldman WJ, Sedmak DD (1999) Human

cytomegalovirus inhibits IFN-alpha-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-alpha signal transduction. J Immunol 162: 6107–6113.

43. Zhu H, Cong JP, Shenk T (1997) Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. Proc Natl Acad Sci USA 94: 13985–13990.

44. Zhu H, Cong JP, Mamtora G, Gingeras T, Shenk T (1998) Cellular gene expression altered by human cytomegalovirus: global monitoring with oligonucleotide arrays. Proc Natl Acad Sci USA 95: 14470–14475.

45. Boyle KA, Pietropaolo RL, Compton T (1999) Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. Molecular and Cellular Biology 19: 3607–3613.

46. Simmen KA, Singh J, Luukkonen BG, Lopper M, Bittner A, et al. (2001) Global modulation of cellular transcription by human cytomegalovirus is initiated by viral glycoprotein B. Proc Natl Acad Sci USA 98: 7140–7145. doi:10.1073/pnas.121177598.

47. Browne EP, Shenk T (2003) Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. Proc Natl Acad Sci USA 100: 11439–11444. doi:10.1073/pnas.1534570100.

48. Abate DA, Watanabe S, Mocarski ES (2004) Major Human Cytomegalovirus Structural Protein pp65 (ppUL83) Prevents Interferon Response Factor 3 Activation in the Interferon Response. Journal of Virology 78: 10995–11006. doi:10.1128/JVI.78.20.10995-11006.2004.

49. DeFilippis VR, Alvarado D, Sali T, Rothenburg S, Fruh K (2009) Human Cytomegalovirus Induces the Interferon Response via the DNA Sensor ZBP1. Journal of Virology 84: 585–598. doi:10.1128/JVI.01748-09.

50. Le VTK, Trilling M, Wilborn M, Hengel H, Zimmermann A (2008) Human cytomegalovirus interferes with signal transducer and activator of transcription (STAT) 2 protein stability and tyrosine phosphorylation. Journal of General Virology 89: 2416–2426. doi:10.1099/vir.0.2008/001669-0.

51. Krauss S, Kaps J, Czech N, Paulus C, Nevels M (2009) Physical Requirements and Functional Consequences of Complex Formation between the Cytomegalovirus IE1 Protein and Human STAT2. Journal of Virology 83: 12854–12870. doi:10.1128/JVI.01164-09.

52. Streblow DN, Nelson JA (2003) Models of HCMV latency and reactivation. Trends Microbiol 11: 293–295.

53. Mocarski ES (2004) Immune escape and exploitation strategies of cytomegaloviruses: impact on and imitation of the major histocompatibility system. Cell Microbiol 6: 707–717. doi:10.1111/j.1462-5822.2004.00425.x.

54. Mocarski ES (2002) Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. Trends Microbiol 10: 332–339.

55. Lin A, Xu H, Yan W (2007) Modulation of HLA expression in human cytomegalovirus

immune evasion. Cell Mol Immunol 4: 91–98.

56. Tomasec P, Braud VM, Rickards C, Powell MB, McSharry BP, et al. (2000) Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. Science 287: 1031.

57. Prod'homme V, Tomasec P, Cunningham C, Lemberg MK, Stanton RJ, et al. (2012) Human Cytomegalovirus UL40 Signal Peptide Regulates Cell Surface Expression of the NK Cell Ligands HLA-E and gpUL18. The Journal of Immunology 188: 2794–2804. doi:10.4049/jimmunol.1102068.

58. Rölle A, Mousavi-Jazi M, Eriksson M, Odeberg J, Söderberg-Nauclér C, et al. (2003) Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. J Immunol 171: 902–908.

59. Dunn C, Chalupny NJ, Sutherland CL, Dosch S, Sivakumar PV, et al. (2003) Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. J Exp Med 197: 1427–1439. doi:10.1084/jem.20022059.

60. Odeberg J, Browne H, Metkar S, Froelich CJ, Brandén L, et al. (2003) The human cytomegalovirus protein UL16 mediates increased resistance to natural killer cell cytotoxicity through resistance to cytolytic proteins. Journal of Virology 77: 4539–4545.

61. Valés-Gómez M, Browne H, Reyburn HT (2003) Expression of the UL16 glycoprotein of Human Cytomegalovirus protects the virus-infected cell from attack by natural killer cells. BMC Immunol 4: 4.

62. Michelson S (2004) Consequences of human cytomegalovirus mimicry. Hum Immunol 65: 465–475. doi:10.1016/j.humimm.2004.02.002.

63. Jurak I, Brune W (2006) Induction of apoptosis limits cytomegalovirus cross-species infection. The EMBO Journal 25: 2634–2642. doi:10.1038/sj.emboj.7601133.

64. Yoshimura A, Ohkubo T, Kiguchi T, Jenkins NA, Gilbert DJ, et al. (1995) A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. The EMBO Journal 14: 2816–2826.

65. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, et al. (1997) Structure and function of a new STAT-induced STAT inhibitor. Nature 387: 924–929. doi:10.1038/43219.

66. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, et al. (1997) A new protein containing an SH2 domain that inhibits JAK kinases. Nature 387: 921–924. doi:10.1038/43213.

67. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, et al. (1997) A family of cytokine-inducible inhibitors of signalling. Nature 387: 917–921. doi:10.1038/43206.

68. Minamoto S, Ikegame K, Ueno K, Narazaki M, Naka T, et al. (1997) Cloning and functional analysis of new members of STAT induced STAT inhibitor (SSI) family: SSI-2 and SSI-3. Biochemical and Biophysical Research Communications 237: 79–

83. doi:10.1006/bbrc.1997.7080.

69. Hilton DJ, Richardson RT, Alexander WS, Viney EM, Willson TA, et al. (1998) Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc Natl Acad Sci USA 95: 114–119.

70. Chen XP, Losman JA, Rothman P (2000) SOCS proteins, regulators of intracellular signaling. Immunity 13: 287–290.

71. Alexander WS (2002) Suppressors of cytokine signalling (SOCS) in the immune system. Nat Rev Immunol 2: 410–416. doi:10.1038/nri818.

72. Matsumoto A, Seki Y, Kubo M, Ohtsuka S, Suzuki A, et al. (1999) Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. Molecular and Cellular Biology 19: 6396–6407.

73. Yoshimura A (2009) Regulation of cytokine signaling by the SOCS and Spred family proteins. Keio J Med 58: 73–83.

74. Akhtar LN, Benveniste EN (2011) Viral Exploitation of Host SOCS Protein Functions. Journal of Virology 85: 1912–1921. doi:10.1128/JVI.01857-10.

75. Croker BA, Kiu H, Nicholson SE (2008) SOCS regulation of the JAK/STAT signalling pathway. Seminars in Cell & Developmental Biology 19: 414–422. doi:10.1016/j.semcdb.2008.07.010.

76. Kile BT, Schulman BA, Alexander WS, Nicola NA, Martin HME, et al. (2002) The SOCS box: a tale of destruction and degradation. Trends Biochem Sci 27: 235–241.

77. Sullivan A, Lu X (2007) ASPP: a new family of oncogenes and tumour suppressor genes. Br J Cancer 96: 196–200. doi:10.1038/sj.bjc.6603525.

78. Greenhalgh CJ, Miller ME, Hilton DJ, Lund PK (2002) Suppressors of cytokine signaling: Relevance to gastrointestinal function and disease. Gastroenterology 123: 2064–2081. doi:10.1053/gast.2002.37068.

79. Kubo M, Hanada T, Yoshimura A (2003) Suppressors of cytokine signaling and immunity. Nat Immunol 4: 1169–1176. doi:10.1038/ni1012.

80. Scutti JAB, Matsuo AL, Pereira FV, Massaoka MH, Figueiredo CR, et al. (2011) Role of SOCS-1 Gene on Melanoma Cell Growth and Tumor Development. Transl Oncol 4: 101–109.

81. Zhang J, Li H, Yu J-P, Wang SE, Ren X-B (2012) Role of SOCS1 in tumor progression and therapeutic application. Int J Cancer 130: 1971–1980. doi:10.1002/ijc.27318.

82. Horndasch M, Culig Z (2011) SOCS-3 antagonizes pro-apoptotic effects of TRAIL and resveratrol in prostate cancer cells. Prostate: n/a–n/a. doi:10.1002/pros.21353.

83. Yoshimura A, Naka T, Kubo M (2007) SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7: 454–465. doi:10.1038/nri2093.

84. Dimitriou ID, Clemenza L, Scotter AJ, Chen G, Guerra FM, et al. (2008) Putting out the fire: coordinated suppression of the innate and adaptive immune systems by

SOCS1 and SOCS3 proteins. Immunol Rev 224: 265–283. doi:10.1111/j.1600-065X.2008.00659.x.

85. Yoshimura A (2012) SOCS, inflammation, and autoimmunity: 1–9. doi:10.3389/fimmu.2012.00020/abstract.

86. Tamiya T, Kashiwagi I, Takahashi R, Yasukawa H, Yoshimura A (2011) Suppressors of Cytokine Signaling (SOCS) Proteins and JAK/STAT Pathways: Regulation of T-Cell Inflammation by SOCS1 and SOCS3. Arteriosclerosis, Thrombosis, and Vascular Biology 31: 980–985. doi:10.1161/ATVBAHA.110.207464.

87. Naka T, Matsumoto T, Narazaki M, Fujimoto M, Morita Y, et al. (1998) Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice. Proc Natl Acad Sci USA 95: 15577–15582.

88. Starr R, Metcalf D, Elefanty AG, Brysha M, Willson TA, et al. (1998) Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. Proc Natl Acad Sci USA 95: 14395–14399.

89. Alexander WS, Starr R, Fenner JE, Scott CL, Handman E, et al. (1999) SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. Cell 98: 597–608.

90. Marine JC, Topham DJ, McKay C, Wang D, Parganas E, et al. (1999) SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. Cell 98: 609–616.

91. Bullen DV, Darwiche R, Metcalf D, Handman E, Alexander WS (2001) Neutralization of interferon-gamma in neonatal SOCS1-/- mice prevents fatty degeneration of the liver but not subsequent fatal inflammatory disease. Immunology 104: 92–98.

92. Metcalf D, Mifsud S, Di Rago L, Nicola NA, Hilton DJ, et al. (2002) Polycystic kidneys and chronic inflammatory lesions are the delayed consequences of loss of the suppressor of cytokine signaling-1 (SOCS-1). Proc Natl Acad Sci USA 99: 943–948. doi:10.1073/pnas.022628499.

93. Tan JC, Rabkin R (2005) Suppressors of cytokine signaling in health and disease. Pediatr Nephrol 20: 567–575. doi:10.1007/s00467-004-1766-8.

94. Metcalf D, Mifsud S, Di Rago L, Alexander WS (2003) The lethal effects of transplantation of Socs1-/- bone marrow cells into irradiated adult syngeneic recipients. Proc Natl Acad Sci USA 100: 8436–8441. doi:10.1073/pnas.1032925100.

95. Saito H, Morita Y, Fujimoto M, Narazaki M, Naka T, et al. (2000) IFN regulatory factor-1-mediated transcriptional activation of mouse STAT-induced STAT inhibitor-1 gene promoter by IFN-gamma. J Immunol 164: 5833–5843.

96. Gregorieff A (2000) Regulation of SOCS-1 Expression by Translational Repression. Journal of Biological Chemistry 275: 21596–21604. doi:10.1074/jbc.M910087199.

97. Schlüter G, Boinska D, Nieman-Seyde SC (2000) Evidence for translational repression of the SOCS-1 major open reading frame by an upstream open reading frame. Biochemical and Biophysical Research Communications 268: 255–261. doi:10.1006/bbrc.2000.2109.

98. Lu C, Huang X, Zhang X, Roensch K, Cao Q, et al. (2011) miR-221 and miR-155 regulate human dendritic cell development, apoptosis, and IL-12 production through targeting of p27kip1, KPC1, and SOCS-1. Blood 117: 4293–4303. doi:10.1182/blood-2010-12-322503.

99. Jiang S, Zhang H-W, Lu M-H, He X-H, Li Y, et al. (2010) MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. Cancer Research 70: 3119–3127. doi:10.1158/0008-5472.CAN-09-4250.

100. Cardoso AL, Guedes JR, Pereira de Almeida L, Pedroso de Lima MC (2011) miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. Immunology 135: 73–88. doi:10.1111/j.1365-2567.2011.03514.x.

101. di Iasio MG, Norcio A, Melloni E, Zauli G (2012) SOCS1 is significantly up-regulated in Nutlin-3-treated p53wild-type B chronic lymphocytic leukemia (B-CLL) samples and shows an inverse correlation with miR-155. Invest New Drugs. doi:10.1007/s10637-011-9786-2.

102. Wang P, Hou J, Lin L, Wang C, Liu X, et al. (2010) Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. The Journal of Immunology 185: 6226–6233. doi:10.4049/jimmunol.1000491.

103. Su C, Hou Z, Zhang C, Tian Z, Zhang J (2011) Ectopic expression of microRNA-155 enhances innate antiviral immunity against HBV infection in human hepatoma cells. Virol J 8: 354. doi:10.1186/1743-422X-8-354.

104. Tili E, Michaille J-J, Cimino A, Costinean S, Dumitru CD, et al. (2007) Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179: 5082–5089.

105. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, et al. (2009) MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci USA 106: 2735–2740. doi:10.1073/pnas.0811073106.

106. O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007) MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci USA 104: 1604–1609. doi:10.1073/pnas.0610731104.

107. Kutty RK, Nagineni CN, Samuel W, Vijayasarathy C, Hooks JJ, et al. (2010) Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment epithelial cells by activating JAK/STAT pathway. Biochemical and Biophysical Research Communications 402: 390–395. doi:10.1016/j.bbrc.2010.10.042.

108. Forrest ARR, Kanamori-Katayama M, Tomaru Y, Lassmann T, Ninomiya N, et al. (2009) Induction of microRNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. Leukemia 24: 460–466. doi:10.1038/leu.2009.246.

109. Hanada T, Yoshida T, Kinjyo I, Minoguchi S, Yasukawa H, et al. (2001) A mutant form of JAB/SOCS1 augments the cytokine-induced JAK/STAT pathway by accelerating degradation of wild-type JAB/CIS family proteins through the SOCS-

box. J Biol Chem 276: 40746–40754. doi:10.1074/jbc.M106139200.

110. Kamura T, Sato S, Haque D, Liu L, Kaelin WGJ, et al. (1998) The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. Genes & Development 12: 3872–3881. Available:http://www.genesdev.org/cgi/doi/10.1101/gad.12.24.3872.

111. Narazaki M, Fujimoto M, Matsumoto T, Morita Y, Saito H, et al. (1998) Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling. Proc Natl Acad Sci USA 95: 13130–13134.

112. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, et al. (1999) The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. Proc Natl Acad Sci USA 96: 2071–2076.

113. Chen XP, Losman JA, Cowan S, Donahue E, Fay S, et al. (2002) Pim serine/threonine kinases regulate the stability of Socs-1 protein. Proc Natl Acad Sci USA 99: 2175–2180. doi:10.1073/pnas.042035699.

114. Roberts AW, Robb L, Rakar S, Hartley L, Cluse L, et al. (2001) Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. Proc Natl Acad Sci USA 98: 9324–9329. doi:10.1073/pnas.161271798.

115. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, et al. (2003) SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. The EMBO Journal 22: 372–384. doi:10.1093/emboj/cdg057.

116. Croker BA, Krebs DL, Zhang J-G, Wormald S, Willson TA, et al. (2003) SOCS3 negatively regulates IL-6 signaling in vivo. Nat Immunol 4: 540–545. doi:10.1038/ni931.

117. Auernhammer CJ, Bousquet C, Melmed S (1999) Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. Proc Natl Acad Sci USA 96: 6964–6969.

118. Lejeune D, Demoulin JB, Renauld JC (2001) Interleukin 9 induces expression of three cytokine signal inhibitors: cytokine-inducible SH2-containing protein, suppressor of cytokine signalling (SOCS)-2 and SOCS-3, but only SOCS-3 overexpression suppresses interleukin 9 signalling. Biochem J 353: 109–116.

119. Ehlting C, Häussinger D, Bode JG (2005) Sp3 is involved in the regulation of SOCS3 gene expression. Biochem J 387: 737–745. doi:10.1042/BJ20041101.

120. Udvadia AJ, Templeton DJ, Horowitz JM (1995) Functional interactions between the retinoblastoma (Rb) protein and Sp-family members: superactivation by Rb requires amino acids necessary for growth suppression. Proc Natl Acad Sci USA 92: 3953–3957.

121. Paul C, Seiliez I, Thissen JP, Le Cam A (2000) Regulation of expression of the rat SOCS-3 gene in hepatocytes by growth hormone, interleukin-6 and glucocorticoids mRNA analysis and promoter characterization. Eur J Biochem 267: 5849–5857.

122. Sasaki A (2002) The N-terminal Truncated Isoform of SOCS3 Translated from an Alternative Initiation AUG Codon under Stress Conditions Is Stable Due to the Lack

of a Major Ubiquitination Site, Lys-6. Journal of Biological Chemistry 278: 2432–2436. doi:10.1074/jbc.C200608200.

123. Haan S (2003) Tyrosine Phosphorylation Disrupts Elongin Interaction and Accelerates SOCS3 Degradation. Journal of Biological Chemistry 278: 31972–31979. doi:10.1074/jbc.M303170200.

124. Babon JJ, Sabo JK, Zhang J-G, Nicola NA, Norton RS (2009) The SOCS Box Encodes a Hierarchy of Affinities for Cullin5: Implications for Ubiquitin Ligase Formation and Cytokine Signalling Suppression. Journal of Molecular Biology 387: 162–174. doi:10.1016/j.jmb.2009.01.024.

125. Babon JJ, McManus EJ, Yao S, DeSouza DP, Mielke LA, et al. (2006) The Structure of SOCS3 Reveals the Basis of the Extended SH2 Domain Function and Identifies an Unstructured Insertion That Regulates Stability. Molecular Cell 22: 205–216. doi:10.1016/j.molcel.2006.03.024.

126. Rechsteiner M, Rogers SW (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci 21: 267–271.

127. Jegalian AG, Wu H (2002) Differential roles of SOCS family members in EpoR signal transduction. J Interferon Cytokine Res 22: 853–860. doi:10.1089/107999002760274863.

128. Greenhalgh CJ, Bertolino P, Asa SL, Metcalf D, Corbin JE, et al. (2002) Growth enhancement in suppressor of cytokine signaling 2 (SOCS-2)-deficient mice is dependent on signal transducer and activator of transcription 5b (STAT5b). Mol Endocrinol 16: 1394–1406.

129. Favre H, Benhamou A, Finidori J, Kelly PA, Edery M (1999) Dual effects of suppressor of cytokine signaling (SOCS-2) on growth hormone signal transduction. FEBS Lett 453: 63–66.

130. Greenhalgh CJ, Metcalf D, Thaus AL, Corbin JE, Uren R, et al. (2002) Biological evidence that SOCS-2 can act either as an enhancer or suppressor of growth hormone signaling. J Biol Chem 277: 40181–40184. doi:10.1074/jbc.C200450200.

131. Metcalf D, Greenhalgh CJ, Viney E, Willson TA, Starr R, et al. (2000) Gigantism in mice lacking suppressor of cytokine signalling-2. Nature 405: 1069–1073. doi:10.1038/35016611.

132. Kario E (2005) Suppressors of Cytokine Signaling 4 and 5 Regulate Epidermal Growth Factor Receptor Signaling. Journal of Biological Chemistry 280: 7038–7048. doi:10.1074/jbc.M408575200.

133. Nicholson SE, Willson TA, Farley A, Starr R, Zhang JG, et al. (1999) Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. The EMBO Journal 18: 375–385. doi:10.1093/emboj/18.2.375.

134. Krebs DL, Uren RT, Metcalf D, Rakar S, Zhang J-G, et al. (2002) SOCS-6 binds to insulin receptor substrate 4, and mice lacking the SOCS-6 gene exhibit mild growth retardation. Molecular and Cellular Biology 22: 4567–4578.

135. Li L, Grønning LM, Anderson PO, Li S, Edvardsen K, et al. (2004) Insulin induces

SOCS-6 expression and its binding to the p85 monomer of phosphoinositide 3-kinase, resulting in improvement in glucose metabolism. J Biol Chem 279: 34107–34114. doi:10.1074/jbc.M312672200.

136. Moore EC, Barber J, Tripp RA (2008) Respiratory syncytial virus (RSV) attachment and nonstructural proteins modify the type I interferon response associated with suppressor of cytokine signaling (SOCS) proteins and IFN-stimulated gene-15 (ISG15). Virol J 5: 116. doi:10.1186/1743-422X-5-116.

137. Miller RC, Schlaepfer E, Baenziger S, Crameri R, Zeller S, et al. (2011) HIV interferes with SOCS-1 and -3 expression levels driving immune activation. Eur J Immunol 41: 1058–1069. doi:10.1002/eji.201041198.

138. Subramanya S, Armant M, Salkowitz JR, Nyakeriga AM, Haridas V, et al. (2010) Enhanced induction of HIV-specific cytotoxic T lymphocytes by dendritic cell-targeted delivery of SOCS-1 siRNA. Mol Ther 18: 2028–2037. doi:10.1038/mt.2010.148.

139. Michaud F, Coulombe F, Gaudreault E, Paquet-Bouchard C, Rola-Pleszczynski M, et al. (2010) Epstein-Barr Virus Interferes with the Amplification of IFNα Secretion by Activating Suppressor of Cytokine Signaling 3 in Primary Human Monocytes. PLoS ONE 5: e11908. doi:10.1371/journal.pone.0011908.t001.

140. Frey KG, Ahmed CMI, Dabelic R, Jager LD, Noon-Song EN, et al. (2009) HSV-1-Induced SOCS-1 Expression in Keratinocytes: Use of a SOCS-1 Antagonist to Block a Novel Mechanism of Viral Immune Evasion. The Journal of Immunology 183: 1253–1262. doi:10.4049/jimmunol.0900570.

141. Yokota S-I, Yokosawa N, Okabayashi T, Suzutani T, Miura S, et al. (2004) Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway. Journal of Virology 78: 6282–6286. doi:10.1128/JVI.78.12.6282-6286.2004.

142. YOKOTA S, YOKOSAWA N, OKABAYASHI T, SUZUTANI T, FUJII N (2005) Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 confers efficient viral replication. Virology 338: 173–181. doi:10.1016/j.virol.2005.04.028.

143. Mahller YY, Sakthivel B, Baird WH, Aronow BJ, Hsu Y-H, et al. (2008) Molecular analysis of human cancer cells infected by an oncolytic HSV-1 reveals multiple upregulated cellular genes and a role for SOCS1 in virus replication. Cancer Gene Ther 15: 733–741. doi:10.1038/cgt.2008.40.

144. Koeberlein B, Hausen zur A, Bektas N, Zentgraf H, Chin R, et al. (2010) Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver. Virus Res 148: 51–59. doi:10.1016/j.virusres.2009.12.003.

145. Bock C-T, Toan NL, Koeberlein B, Song LH, Chin R, et al. (2008) Subcellular mislocalization of mutant hepatitis B X proteins contributes to modulation of STAT/SOCS signaling in hepatocellular carcinoma. Intervirology 51: 432–443. doi:10.1159/000209672.

146. Ye L, Wang X, Metzger DS, Riedel E, Montaner LJ, et al. (2010) Upregulation of SOCS-3 and PIAS-3 Impairs IL-12-Mediated Interferon-Gamma Response in CD56+

T Cells in HCV-Infected Heroin Users. PLoS ONE 5: e9602. doi:10.1371/journal.pone.0009602.t002.

147. MIYOSHI H, FUJIE H, SHINTANI Y, TSUTSUMI T, SHINZAWA S, et al. (2005) Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling gene expression. Journal of Hepatology 43: 757–763. doi:10.1016/j.jhep.2005.03.028.

148. Pazienza V, Vinciguerra M, Andriulli A, Mangia A (2010) Hepatitis C virus core protein genotype 3a increases SOCS-7 expression through PPAR- in Huh-7 cells. Journal of General Virology 91: 1678–1686. doi:10.1099/vir.0.020644-0.

149. Pauli E-K, Schmolke M, Wolff T, Viemann D, Roth J, et al. (2008) Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression. PLoS Pathog 4: e1000196. doi:10.1371/journal.ppat.1000196.

150. Hashimoto K, Ishibashi K, Ishioka K, Zhao D, Sato M, et al. (2009) RSV replication is attenuated by counteracting expression of the suppressor of cytokine signaling (SOCS) molecules. Virology 391: 162–170. doi:10.1016/j.virol.2009.06.026.

151. Yadav A, Fitzgerald P, Sajadi MM, Gilliam B, Lafferty MK, et al. (2009) Increased expression of suppressor of cytokine signaling-1 (SOCS-1): A mechanism for dysregulated T helper-1 responses in HIV-1 disease. Virology 385: 126–133. doi:10.1016/j.virol.2008.11.039.

152. Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, et al. (1994) Functional role of type I and type II interferons in antiviral defense. Science 264: 1918–1921.

153. Lo AKF, Lo KW, Tsao SW, Wong HL, Hui JWY, et al. (2006) Epstein-Barr Virus Infection Alters Cellular Signal Cascades in Human Nasopharyngeal Epithelial Cells. NEO 8: 173–180. doi:10.1593/neo.05625.

154. Nachtwey J, Spencer JV (2008) HCMV IL-10 Suppresses Cytokine Expression in Monocytes Through Inhibition of Nuclear Factor- κB. Viral Immunology 21: 477–482. doi:10.1089/vim.2008.0048.

155. Carlier J, Martin H, Mariame B, Rauwel B, Mengelle C, et al. (2011) Paracrine inhibition of GM-CSF signaling by human cytomegalovirus in monocytes differentiating to dendritic cells. Blood 118: 6783–6792. doi:10.1182/blood-2011-02-337956.

156. Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, et al. (2007) Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J Immunol 179: 5966–5976.

157. Qin H, Wilson CA, Roberts KL, Baker BJ, Zhao X, et al. (2006) IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. J Immunol 177: 7761–7771.

158. Niemand C, Nimmesgern A, Haan S, Fischer P, Schaper F, et al. (2003) Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. J Immunol 170: 3263–3272.

159. Delgado-Ortega M, Melo S, Meurens F (2011) Expression of SOCS1-7 and CIS mRNA in porcine tissues. Veterinary Immunology and Immunopathology: 1–6.

doi:10.1016/j.vetimm.2011.08.002.

160. Royaee AR, Husmann RJ, Dawson HD, Calzada-Nova G, Schnitzlein WM, et al. (2004) Deciphering the involvement of innate immune factors in the development of the host response to PRRSV vaccination. Veterinary Immunology and Immunopathology 102: 199–216. doi:10.1016/j.vetimm.2004.09.018.

161. Wysocki M, Chen H, Steibel JP, Kuhar D, Petry D, et al. (2011) Identifying putative candidate genes and pathways involved in immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Animal Genetics 43: 328–332. doi:10.1111/j.1365-2052.2011.02251.x.

162. Uthe JJ, Royaee A, Lunney JK, Stabel TJ, Zhao S-H, et al. (2007) Porcine differential gene expression in response to Salmonella enterica serovars Choleraesuis and Typhimurium. Molecular Immunology 44: 2900–2914. doi:10.1016/j.molimm.2007.01.016.

163. Du B-W, Yang G-S, Sun C (2007) [Cloning and sequence analysis of SOCS-2 gene in pig]. Sheng Wu Gong Cheng Xue Bao 23: 1091–1096.

164. Bruel TE, Guibon R, Melo S, n NG, Salmon H, et al. (2010) Epithelial induction of porcine suppressor of cytokine signaling 2 (SOCS2) gene expression in response to Entamoeba histolytica. 34: 562–571. doi:10.1016/j.dci.2009.12.017.

165. Weber TE, Kerr BJ (2006) Butyrate differentially regulates cytokines and proliferation in porcine peripheral blood mononuclear cells. Veterinary Immunology and Immunopathology 113: 139–147. doi:10.1016/j.vetimm.2006.04.006.

166. Orth JHC, Aktories K, Kubatzky KF (2007) Modulation of host cell gene expression through activation of STAT transcription factors by Pasteurella multocida toxin. J Biol Chem 282: 3050–3057. doi:10.1074/jbc.M609018200.

167. WU J, WANG B, ZHANG H, YU T, YANG G (2007) Different Transcription Profiles of SOCS-3, ob and IGF-I Genes and their Possible Correlations in Obese and Lean Pigs. Acta Biochim Biophys Sinica 39: 305–310. doi:10.1111/j.1745-7270.2007.00274.x.

168. ZHANG H, WU J, WANG B, Lü Z, YANG G (2008) [Regulation of SOCS-3, OB, GLUT4 and PPARgamma gene expression by insulin and dexamethasone in porcine primary adipocyte]. Sheng Wu Gong Cheng Xue Bao 24: 1354–1360.

169. Ghielmetti M (2009) Human Leukocyte Recruitment to the Porcine Endothelium: Implications for Xenotransplantation. Thesis Nr. 18383, Swiss Federal Institute of Technology Zurich, Switzerland

170. Seebach JD, Schneider MK, Comrack CA, LeGuern A, Kolb SA, et al. (2001) Immortalized bone-marrow derived pig endothelial cells. Xenotransplantation 8: 48–61.

171. Terstegen L, Maassen BG, Radtke S, Behrmann I, Schaper F, et al. (2000) Differential inhibition of IL-6-type cytokine-induced STAT activation by PMA. FEBS Lett 478: 100–104.

172. Naldini L, Blömer U, Gage FH, Trono D, Verma IM (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains

injected with a lentiviral vector. Proc Natl Acad Sci USA 93: 11382–11388.

173. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, et al. (1998) A third-generation lentivirus vector with a conditional packaging system. Journal of Virology 72: 8463–8471.

174. Song MM, Shuai K (1998) The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. J Biol Chem 273: 35056–35062.

175. Koeberlein B, Hausen AZ, Bektas N, Zentgraf H, Chin R, et al. (2010) Hepatitis B virus overexpresses suppressor of cytokine signaling-3 (SOCS3) thereby contributing to severity of inflammation in the liver. Virus Res 148: 51–59. doi:10.1016/j.virusres.2009.12.003.

176. Benz C, Reusch U, Muranyi W, Brune W, Atalay R, et al. (2001) Efficient downregulation of major histocompatibility complex class I molecules in human epithelial cells infected with cytomegalovirus. J Gen Virol 82: 2061–2070.

177. del Val M, Hengel H, Häcker H, Hartlaub U, Ruppert T, et al. (1992) Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. J Exp Med 176: 729–738.

178. Beersma MF, Bijlmakers MJ, Ploegh HL (1993) Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains. J Immunol 151: 4455–4464.

179. Cebulla CM, Miller DM, Zhang Y, Rahill BM, Zimmerman P, et al. (2002) Human cytomegalovirus disrupts constitutive MHC class II expression. J Immunol 169: 167–176.

180. Lu F, Weidmer A, Liu CG, Volinia S, Croce CM, et al. (2008) Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. Journal of Virology 82: 10436–10443. doi:10.1128/JVI.00752-08.

181. Skalsky RL, Samols MA, Plaisance KB, Boss IW, Riva A, et al. (2007) Kaposi's Sarcoma-Associated Herpesvirus Encodes an Ortholog of miR-155. Journal of Virology 81: 12836–12845. doi:10.1128/JVI.01804-07.

182. Paulus C, Krauss S, Nevels M (2006) A human cytomegalovirus antagonist of type I IFN-dependent signal transducer and activator of transcription signaling. Proc Natl Acad Sci USA 103: 3840–3845. doi:10.1073/pnas.0600007103.

183. Sinzger C, Schmidt K, Knapp J, Kahl M, Beck R, et al. (1999) Modification of human cytomegalovirus tropism through propagation in vitro is associated with changes in the viral genome. J Gen Virol 80 (Pt 11): 2867–2877.

184. Jacobs JP, Jones CM, Baille JP (1970) Characteristics of a human diploid cell designated MRC-5. Nature 227: 168–170.

185. Mahy BWJ (1996) Virology methods manual. Academic Pr. pp.

186. Bossart W, Nuss DL, Paoletti E (1978) Effect of UV irradiation on the expression of vaccinia virus gene products synthesized in a cell-free system coupling transcription

and translation. Journal of Virology 26: 673–680.

187. Watson S, Mercier S, Bye C, Wilkinson J, Cunningham A, et al. (2007) Determination of Suitable Housekeeping Genes for Normalisation of Quantitative Real Time PCR Analysis of Cells Infected with Human Immunodeficiency Virus and Herpes Viruses. Virol J 4: 130. doi:10.1186/1743-422X-4-130.

188. Ekser B, Ezzelarab M, Hara H, van der Windt DJ, Wijkstrom M, et al. (2012) Clinical xenotransplantation: the next medical revolution? Lancet 379: 672–683. doi:10.1016/S0140-6736(11)61091-X.

189. Garkavenko O, Durbin K, Tan P, Elliott R (2011) Islets transplantation: New Zealand experience. Xenotransplantation 18: 60–60. doi:10.1111/j.1399-3089.2010.00607_3.x.

190. Yamada K, Scalea J (2012) Current progress in xenogeneic tolerance. Curr Opin Organ Transplant 17: 168–173. doi:10.1097/MOT.0b013e32835090f6.

191. Fishman JA, Scobie L, Takeuchi Y (2012) Xenotransplantation-associated infectious risk: a WHO consultation. Xenotransplantation 19: 72–81. doi:10.1111/j.1399-3089.2012.00693.x.

192. Oh H-Y, Jin X, Kim J-G, Oh M-J, Pian X, et al. (2007) Characteristics of primary and immortalized fibroblast cells derived from the miniature and domestic pigs. BMC Cell Biol 8: 20. doi:10.1186/1471-2121-8-20.

193. Cheng J, Zhang D, Zhou C, Marasco WA (2004) Down-regulation of SHP1 and up-regulation of negative regulators of JAK/STAT signaling in HTLV-1 transformed cell lines and freshly transformed human peripheral blood CD4+ T-Cells. Leukemia Research 28: 71–82. doi:10.1016/S0145-2126(03)00158-9.

194. Freshney RI (2011) Culture of Animal Cells. Hoboken, NJ, USA: John Wiley & Sons, Inc. pp. doi:10.1002/9780470649367.ch17.

195. Zhang S, Wang W, Wang E, Qiu X (2012) SOCS3 expression is inversely correlated with Pyk2 in non-small cell lung cancer and exogenous SOCS3 inhibits proliferation and invasion of A549 cells. Pathology. doi:10.1097/PAT.0b013e328354ffdf.

196. SCHERER WFW, SYVERTON JTJ, GEY GOG (1953) Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J Exp Med 97: 695–710.

197. Sakoda T, Kasahara N, Kedes L, Ohyanagi M (2007) Lentiviral Vector-Mediated Gene Transfer to Endotherial Cells Compared with Adenoviral and Retroviral Vectors. Preparative Biochemistry and Biotechnology 37: 1–11. doi:10.1080/10826060601039345.

8. List of abbreviations

2A2	bone marrow derived endothelial cells
BSA	bovine serum albumin
CIS	cytokine-inducible SH2
CMV-MoDCs	CMV-infected MoDCs
CNTF	ciliary neuronotrophic factor
DNAM-1	DNAX accessory molecule-1
E	early
EBV	Epstein Barr virus
EC	endothelial cells
EGF	epidermal growth factor
EPO	erythropoietin
Fw.	Forward
DT	double transfected
g	glycoprotein
G-CSF	granulocyte colony stimulation factor
GFP	green fluorescence protein
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
HAEC	primary aortic endothelial cells
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HMVEC	human primary lung-derived endothelial cells
HSV	herpes simplex virus
HUVEC	human umbilical vein endothelial cells
IFN	interferon
IGF-1	insulin-like growth factor-1
IRF9	IFN regulatory factor-9
IE	immediate early
IL	interleukin
JAK	janus kinase
KSHV	Kaposi's sarcoma-associated herpes virus
KIR	kinase inhibitor region
L	late
LB	lysis buffer
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
M-CSF	macrophage-colony stimulating factor
MHC	major histocompatibility complex
mIR-155	microRNA-155
MoDCs	monocytes-derived dendritic cells
MOI	multiplicity of infection
MRC5	human lung derived fibroblast
Mx	myxovirus resistance
NK	natural killer
NKG2D	natural killer group 2 member D
N.T.	non-transfected
PEDSV.15	endothelial cells from aortic origin
PAEC-KO	porcine aortic EC knock-out for α1,3 galactosyltransferase
PAMPs	pathogen-associated molecular patterns
pEC	porcine endothelial cells
PEST	Proline, Glutamate, Aspartate, Serine and Threonine
PMA	phorbol 12-myristate 13-acetate

p.i.	post infection
p.s.	post stimulation
RT-PCR	real time polymerase chain reaction
Rv.	Reverse
SD	standard deviation
SDS-Gel	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	short interfering RNA
siCNTR	silenced control
siSOCS	silenced SOCS
SOCS	suppressor of cytokine signaling
$SOCS^{-/-}$	SOCS deficient mice
ST	single transfected
STAT	signal transducer and activator of transcription
TLR	toll like receptor
TNFα	tumor necrosis factor α
TPO	thrombopoietin
TSH	thyroid stimulating hormone
TSLP	thymic stromal lymphopoietin
US	unique short protein
VSV	vesicular stomatitis virus

9. Curriculum vitae

Surname: Sonzogni
First Name: Olmo
Date of Birth: 05.03.1980
Nationality: Swiss (CH)
Place of Origin: Moleno, TI

Education and Scientific Work Experiences

2008-to date	Ph.D. student at University of Zurich, Suppressors of Cytokine Signaling Proteins 1 and 3 and the Control of Human Cytomegalovirus Infection in Endothelial Cells PhD thesis at the Division of Infectious diseases and Hospital Epidemiology, University Hospital Zurich (Prof. Dr. med. Nicolas J. Mueller) Member of Ph.D. program Life Science Zurich Graduate School; Microbiology and Immunology (MIM)	
2007- 2008	Research Associate, Development of a test to differentiate FIV-infected from FIV-vaccinated cats Clinical Laboratory, Vetsuisse Faculty Zurich, University of Zurich (Prof. Dr. med. vet. Hans Lutz)	
2000-2006	University of Zurich:	**Master of Science**, Diploma in Zoology
	Master thesis:	Analysis of Protein VIII of Adenovirus type 2 Department of Cell Biology, Institute of Zoology, University of Zurich (Prof. Dr. Urs Greber)
	Major Subjects:	**Zoology:** • **Modern Genetics and Genomics** • **Faunistics and Taxonomy of Vertebrates** • **Cell Biology**
	Minor Subjects:	**Molecular Biology** **Anthropology**
	Additional Courses:	**Ethology** **Gene Technology, ETH Zurich**
1995-1999	High School Bellinzona, Type C (Scientific Orientation)	

Scientific Meetings

Oral presentations

Influence of HCMV infection on suppressor of cytokine signaling (SOCS) expression
4th STAX Meeting, November 6, 2009, Bern, Switzerland
Olmo Sonzogni, Maddalena Ghielmetti, Lea Häberli, Mårten Schneider, Roberto Speck, Regina Miller, Joerg D. Seebach, Marek Fischer, Anne-Laure Millard, Nicolas J. Mueller

Influence of human cytomegalovirus infection on suppressor of cytokine signaling (SOCS) expression
XXII. Meeting of the Swiss Immunology Ph.D. Students, March 22. – 24, 2010, Wolfsberg, Switzerland

Poster presentations

Maddalena Ghielmetti, Olmo Sonzogni, Lea Häberli, Anne-Laure Millard, Mårten Schneider, Roberto Speck, Regina Miller, Joerg D. Seebach, Marek Fischer, Nicolas J. Mueller
Suppressor of cytokine signaling transcription in human cytomegalovirus-infected porcine endothelial cells
Joint Meeting IPTTA-IXA, October 12-16, 2009, Venice, Italy

Olmo Sonzogni, Maddalena Ghielmetti, Lea Häberli, Anne-Laure Millard, Mårten Schneider, Roberto Speck, Regina Miller, Joerg D. Seebach, Marek Fischer, Nicolas J. Mueller
Influence of HCMV infection on suppressor of cytokine signaling (SOCS) expression
Second Swiss Workshop in Fundamental Virology, February 1-2, 2010, Gerzensee, Bern, Switzerland

Olmo Sonzogni, Maddalena Ghielmetti, Lea Häberli, Mårten Schneider, Roberto Speck, Regina Miller, Joerg D. Seebach, Marek Fischer, Anne-Laure Millard, Nicolas J. Mueller
Influence of human cytomegalovirus infection on suppressor of cytokine signaling (SOCS) expression
9th Day of Clinical Research, April 08, 2010, Zurich, Switzerland

Olmo Sonzogni, Maddalena Ghielmetti, Lea Häberli, Mårten Schneider, Roberto Speck, Regina Miller, Joerg D. Seebach, Marek Fischer, Anne-Laure Millard, Nicolas J. Mueller
Influence of human cytomegalovirus infection on suppressor of cytokine signaling (SOCS) expression
Annual Congress SGAI/SSAI Translational Immunology, April 15-16, 2010, St Gallen, Switzerland

Olmo Sonzogni, Anne-Laure Millard, Mårten Schneider, Roberto Speck, Marek Fischer, Nicolas J. Mueller
Modulation of suppressor of cytokine signaling 1 and 3 expression in endothelial cells
Annual Congress SGAI/SSAI Translational Immunology, March 17-18, 2011, Lugano, Switzerland

Olmo Sonzogni, Anne-Laure Millard, Mårten Schneider, Roberto Speck, Marek Fischer, Nicolas J. Mueller
Modulation of SOCS1 and SOCS3 leads to impaired human cytomegalovirus infection of human primary endothelial cells
Third Swiss Workshop in Fundamental Virology, August 29-30, 2011, Thun, Switzerland

Publications

Submitted

Suppressor of Cytokine Signaling 3 is a Key Factor for Efficient Cytomegalovirus Replication in Primary Endothelial Cells
Olmo Sonzogni, Anne-Laure Millard, Aline Marie Taveira, Marten K.J. Schneider, Li Duo, Roberto F. Speck, Nicolas J. Mueller

In preparation

Paper on Adenovirus 5 proteome, (Greber's Lab)

Additional Professional Courses

2009
Immunology I, ETH Zurich
Immunology and Infection Biology JC, ETH Zurich

2010
Immunology II, ETH Zurich
Immunology and Infection Biology JC, ETH Zurich

2011
Principles of Biosafety in Medical and Biological Research, University of Zurich

10. Acknowledgements

I first would like to thank Professor Nicolas Müller for giving me the opportunity to carry out my thesis in his lab.

Many thanks are also due to the members of my PhD committee, Professor Christoph Renner and Professor Amedeo Caflisch for helpful discussions and suggestions during our meetings.

Many thanks to the people I worked with, starting with PhD students Aline Marie Taveira and Nicolas Ponroy currently working in the lab, followed by previous coworker Dr. Maddalena Ghielmetti who introduced me to the SOCS's world, and by Dr. Anne-Laure Millard, Lea Häberli and Marten Schneider for helpful discussions. Also acknowledged for their contributions are PD Dr. Jovan Pavlovic, Dr. Thomas Ludersdorfer and Dr. Christian Wisskirchen who kindly provided crucial reagents and materials and many important insights into the Mx world.

It was a pleasure to interact with Prof. Roberto Speck and members of his team, Dr. Regina Miller, Li Duo and Erika Schläpfer-Nadal. Prof. Karrer's group including Vincent Tchang, Dr. Andrea Mekker and Kerstin Wanke as well as PD Dr. Annelies Zinkernagel's group including Dr. Kati Seidl, Andrea Tarnutzer, Nadia Keller, Katrin Schilcher, Claudia Zürcher and Dr. Ulrich Matt were great lab mates. I could count on their ongoing support, nice discussions and fun coffee breaks. A special thank you goes to Dr. Satoshi Uchiyama for being such a nice, quiet and productive person, an example both in and out of the lab. To Fede for her incredible patience and ability to see the good side of everything, and for being such a good friend, GRAZIE!

To my brother and friend Eliano and to Torria for sharing every frustration and success and for supporting me also when it was not easy… To Omar and Guaina, roommates and special friends even now that we are in different countries. To Meui and Claude for the old times and those which have to come! Thank you very much!!

A special thanks to Céline for being such a source of inspiration and strength, without you this work would not have been possible.

Finally, profound gratitude goes to my mom and dad for their encouragements in all the projects I have undertaken. My professional and personal achievements would certainly not have been possible without their love and support. Thank you so much.